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1955



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ON  
GENETIC RECOMBINATION

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## INTRODUCTION

The newer developments in regard to the study of the structure of nucleic acids, especially deoxyribonucleic acids, has made it desirable to try to relate this to the problem of genetic recombination. Progress of the work in bacterial genetics, transforming principle, and transduction — the new approaches which have been developed in the genetic study of higher organisms — have given the investigator new tools to study the problem of recombination.

The meetings of the Seventh Annual Research Conference bore out the timeliness of the subject. This conference, held in Oak Ridge in April 1954, was sponsored by the Biology Division of the Oak Ridge National Laboratory and the Division of Biology and Medicine, U. S. Atomic Energy Commission.

As at previous conferences, free and open discussions were encouraged. An evening was devoted to a discussion on the present status of the role of oxygen in X-ray sensitivity. Papers and discussions in this session are included.

Most of the speakers submitted manuscripts and a large part of the discussion is being reproduced.

Dr. W. K. Baker and Dr. Drew Schwartz were largely responsible for arranging the program.

Previous symposia in this series are:

1948 — Radiation Genetics

1949 — Radiation Microbiology and Biochemistry

1950 — Biochemistry of Nucleic Acids

1951 — Physiological Effects of Radiation at the Cellular Level

1952 — Some Aspects of Microbial Metabolism

1953 — Effects of Radiation and Other Deleterious Agents on Embryonic Development.

ALEXANDER HOLLAENDER





# BACTERIAL TRANSFORMATION

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It will be the intention in this report to discuss the general characteristics of transformation reactions, coming finally to certain recent data which seem to have a more direct bearing on recombination. The transformation reaction, first discovered by Griffith in 1928, and clarified by the classic work reported by Avery, MacLeod, and McCarty ('44), can be defined as a heritable change induced in bacteria by an extract of other bacteria. The extract conveys a part of the genetic endowment of one strain and carries it to another strain. Avery, MacLeod, and McCarty showed that the transforming activity of encapsulated pneumococci was resident in their nucleic acid fraction. It could be recovered as a biochemical preparation of deoxyribonucleic acid (DNA); this material applied to unencapsulated organisms would convert a proportion of them into encapsulated cells having the same serological type as the donor cells.

There are many specific capsule types in *Diplococcus pneumoniae* and the type III capsule is different from all others in that it has certain serological identity. The DNA extract confers this same particular type of encapsulation on another unencapsulated pneumococcus. The fact that each encapsulated organism so produced can be subcultured and made to produce large quantities of progeny all carrying the new type of capsule made the process seem to have genetic significance; but it was not clear at that time what the genetic interpretation might be.

The material conveyed in DNA extract might be the whole genetic endowment of the cell, or only a portion. It might

correspond, in other words, to the nucleus, the chromosome, or a single gene. It could be an inducer, and some geneticists described it as such. It might be thought to be a transferable factor involved directly in specific capsule production.

The DNA's are high molecular materials with molecular weight around one million. Their solutions are viscous, precipitable by alcohol, acetone, or acids, as fibers of characteristic appearance. The biological activity is lost whenever the molecules are degraded by the specific enzyme deoxyribonuclease.

The years since 1944 yielded some twenty-five capsule transformations in *D. pneumoniae* and *Hemophilus*, one in *Escherichia coli*, one or two of meningococci (for reviews, see Austrian, '52; Hotchkiss, '52). There have been a few other antigen transformations. The ones with which we shall mainly be concerned are the drug-resistance transformations which were introduced about three years ago, and which allow an additional step to be made. That additional step is the selection from the transformed culture of those cells which have been changed. For studying a number of questions it is important to work with the transformants separated from the untransformed cells.

Selecting in the drug medium a rare mutant that appears perhaps once in a million or ten million divisions will give a culture of drug-resistant cells. From this, as with the capsule strains, the DNA extract can be prepared and handled chemically, and when added back to drug-sensitive cells, will produce a proportion of drug-resistant cells far larger — easily ten thousand times as large — than the number which are produced by spontaneous mutation (Hotchkiss, '51).

These properties then allow one to treat the final transformed culture with the drug, and thereafter to work only with the transformant cells. This also permits exact quantitation of how many have been formed and approximately at what time they have been altered.

## GENETIC NATURE OF TRANSFORMING UNITS

A first question seems to be: What is the nature of the transferred unit? If it were a bacterial gene, it should first of all behave unitwise. The different capsule factors could be construed as alleles; in any case it is not ordinarily possible to incorporate more than one of such characters in a strain, but having a capsule factor and a drug-resistant factor, one can prepare a doubly marked strain.

An encapsulated and drug-resistant strain gave a DNA extract which now carried two properties and revealed some three years ago that the two properties moved separately into individual transformant cells (Hotchkiss, '51). Those which became encapsulated, in general, did not become drug resistant; and those which became drug resistant, in general, did not become encapsulated. So at least in this case, there was separation back into units that, acquired independently in the donor cell, were now in transformation acting again as units; a finding consistent with the DNA's being analogous to a gene material. Several other pairs of markers behave similarly independently.

The next step was in recognizing certain quantitative characters in the penicillin-resistant scheme. Demerec ('45) has shown that penicillin resistance arrives in *staphylococcus* — and the same can be shown for *pneumococcus* — in a series of stepwise, discrete mutations. These are found in transformation also to be transferred in stepwise fashion.

When *pneumococci* have reached a high, multiple-step level of penicillin resistance, their DNA will transfer to sensitive cells at first only the single property of the first mutation step. In subsequent exposures to the same DNA, the transformants will move stepwise up the ladder of penicillin resistance. The slightly resistant strain produced in the first step can be subsequently retransformed by the same DNA from the same highly resistant strain, moving on to the second step in the genetic history — giving rise to a transformant strain of moderate (two-step) resistance — and so on (Hotchkiss, '51).

This is an indication that transformation is recapitulating the genetic history of the strain, there being transferred, via the DNA, the very material in which the record of the individual mutations is stored. In streptomycin resistance, there is observed a stepwise system which operates just as described for penicillin, but, in addition, an alternative high level of streptomycin resistance acquired as a single step. Accordingly, there may be two types of streptomycin-resistant cells, difficult to distinguish phenotypically — one that has arrived at a high-level resistance in discrete steps, and one that has arrived by a single, big mutational jump. Although phenotypically similar, their DNA's will show in the transformation phenomenon a record of their genetic history. They are found to transfer to sensitive cells the original first resistance step attained by the strain; in one case, the small first step and the other, the full, high-level resistance (Hotchkiss, '52).

Another case is brought out in the work of Marmur in this laboratory on the transformation of the property of mannitol phosphate dehydrogenase in pneumococci (Marmur and Hotchkiss, '55). The property of growing in mannitol is an adaptive process. Mutant cells which have not been pre-adapted to mannitol undergo a long lag period before they are able to utilize this sugar alcohol for growth, or to oxidize it in metabolism experiments. Cells which have been pre-adapted in mannitol will use it immediately.

Unadapted cells do not have enzymes that are able to dehydrogenate mannitol phosphate, but they begin to acquire demonstrable enzyme during adaptation. In both states, the cells are, of course, genetically the same. What is interesting is that transformation by DNA from the two types of genetically identical, but phenotypically different, cells gives the same number of transformants induced over a whole range of DNA concentrations. This again shows that the genetic potentialities of the cell were transferred rather than the attained state, just as the highly drug-resistant cells donated their genetic first step rather than their fully attained state.



These analyses seem to support the early inferences that the determinants being transferred in the DNA transformations are the bacterial genes themselves.

As concerns the nature of the material, the first problem was to show that the transforming extracts actually contained no significant amount of protein, as had already been elegantly indicated in the work of Avery by enzymatic and other evidence. The principal addition since then has been to show that, though earlier analytical data did not exclude a small amount of protein, there was less than 0.02% of protein in a pneumococcus-transforming preparation of typical full activity (Hotchkiss, '52). As its protein content was reduced from a somewhat larger level to this level of below 0.02%, there was no particular change in the biological activity. So, the material, which as indicated before is effectively a bacterial gene preparation, seems to be made up exclusively of DNA.

#### LINKED TRANSFORMING UNITS IN DNA

Of most interest for this symposium are the evidences of a higher order of genetic complexity in the DNA and the nature of the processes instituted by its introduction into the cell. The first evidence that transformation is not always transfer of single units came in relating the mannitol transformation with the streptomycin-resistance transformation by DNA from doubly marked strains.

In a large number of experiments, carried out in collaboration with Marmur and Lane, a rather large proportion, 20–25%, of the *M* transformants, had also acquired streptomycin resistance, *S*. The *MS* cells seemed to be the result of a simultaneous double transformation, since there were so many more than would have been expected on the basis of random successive single transformations (Hotchkiss and Marmur, '54). Thus the frequency of the *MS* transformants from a population *N*, turned out to be five to twenty times as great as the product of the individual frequencies,  $M/N$  times  $S/N$ . Although it might seem to be reasonable to expect

those cells responding to one transforming factor to show more than average frequency of transformation to another factor, actually, interference between DNA's seems to make successive transformations infrequent. When pairs of markers believed not to be associated were used, the number of double transformations was always less than (one-tenth to two-fifths) the number that could have been expected as the result of random single transformation events. One DNA added to another one diminishes the effectiveness of the latter.

Indeed, a mixture of DNA from a mannitol-utilizing strain and that from a streptomycin-resistant strain produces about one-fourth as many *MS* transformants as would be expected by chance alone from the rates of the single transformations (Hotchkiss and Marmur, '54). Thus it can be seen that the frequency of double *MS* transformations is most likely twenty to one hundred times as great as can be accounted for on the basis of single events, when DNA derived from the doubly marked strain is used. It must be concluded that, by reason of coexistence within the same cells, the *M* and the *S* transforming factors are in some way "linked" within the same DNA particle, much as genic factors are considered to be linked within the chromosome as a whole.

It is too early to state with assurance whether the *M* and *S* determinants are linked in all the DNA particles (and become separated 75-80% of the time in a process akin to crossing over during transformation), or whether some proportion, up to 80%, of them become separated during isolation. Inasmuch as no amount of chemical manipulation during preparation of the DNA has altered the degree of linkage, and since spontaneously mutated strains and a whole series of multiply marked strains which acquire the *M* and *S* factors by transformation as well as different combinations of other markers all gave DNA's with similar linkage properties, it seems reasonable to conclude that the relative constancy of the 20-25% linkage is based on the characteristic crossing

over behavior in the recipient cell of DNA particles which may all be essentially alike.

Reported observations seemed to imply the association between different subunits of transforming agents affecting capsule formation. Ephrussi-Taylor ('51a,b) had indicated that there were interactions between the DNA of a donor cell and the determinants of the recipient cell, such that agents controlling quantitatively different degrees of capsular polysaccharide synthesis seem to recombine and thereafter lead to more potent synthesis than either parental strain could support. Since the new potent determinant was found in the DNA, and it seldom if ever was observed to redissociate into biological subunits, a reasonable interpretation seemed to be that a kind of linkage and crossing over govern the outcome of these interactions during transformation. Inasmuch as the inferred crossing over would have to occur within an otherwise distinct unit, and within a mutated region which governed the rate or efficiency of one and the same synthetic process, the system was deemed possibly analogous to a pseudoallelic one. The mixed capsule transformation described for *Hemophilus* (Leidy, Hahn, and Alexander, '53) seems to be an instance in which two wild-type markers affecting the same cell property in different ways have been assembled into either a heterozygotic or recombinant strain from which can be derived a functioning "mixed" transforming agent. The gradual dissociation of the strain itself back into pure encapsulated types, leaves some doubt whether the partial dissociation of the individual markers in a transformation by its partially purified DNA is caused by interactions going on during transformation or perhaps to inherent instability or heterogeneity of the DNA. It seems that the metabolically unrelated pair of mannitol and streptomycin markers of *Pneumococcus* illustrate more simply and safely the existence of associations analogous to classical genetic linkage; they may be taken, however, as indications that a similar mechanism could be assumed for the two cases just described.

## TRANSFORMATION AS GENETIC EXCHANGE

More satisfying evidence that something akin to linkage and crossing over are involved in transformation comes from further developments in the work carried out with Marmur and Lane. What we have called reverse transformations can result in the loss of selective characters in pneumococci (Hotchkiss and Marmur, '54). By taking advantage of the association between *M* and *S* there is a good chance of detecting any reasonably frequent change in *M* during intro-

TABLE 1

*Evidence of linkage between allelic forms of mannitol and streptomycin-resistance characters in pneumococcus*

CHARACTERS PRESENT IN		CHARACTERS FOUND IN TRANSFORMANTS		
Donor DNA	Recipient cell	Single		Double
<i>MS</i>	<i>ms</i>	<i>Ms</i>	<i>mS</i>	Many <i>MS</i>
<i>Ms</i> + <i>mS</i>	<i>ms</i>	<i>Ms</i> <sup>a</sup>	<i>mS</i> <sup>a</sup>	Fewer than random <i>MS</i>
<i>Ms</i>	<i>mS</i>	<i>MS</i>	( <i>ms</i> )	Many <i>Ms</i>
<i>mS</i>	<i>Ms</i>	<i>MS</i>	( <i>ms</i> )	Many <i>mS</i>
<i>MsP</i>	<i>mSP</i>	<i>MSP</i> <sup>a</sup>	<i>msP</i>	Fewer than random <i>MsP</i>

Characters: *M*, mannitol utilizing; *S*, streptomycin resistant; *P*, penicillin resistant; small letters signify absence of corresponding selective property, but do not imply recessiveness relation. Phenotypes in parentheses difficult to detect and not observed.

<sup>a</sup> These genotypes could also result from double transformation.

(Experiments with J. Marmur and D. Lane.)

duction of *S*, or vice versa. The reverse transformations are represented in table 1 in the two rows in which *M*, or *S*, characters preexisting in a recipient cell are indicated as removed during simultaneous transformation to the selective phase of the other, the *S* or the *M* character, respectively. We infer therefrom the existence of the nonselective phases, *m* and *s*, of the same characters. Although the exchange apparently results in the elimination of one nonviable portion of



the determinant material, the reciprocal "crosses" together support the picture of the process as an exchange and indicate also that dominance relations in a diploid state are not involved. The frequency of recovery of doubly transformed strains is indicated in table 2. It will be seen that for every six to seven of the singly transformed cells there was isolated one which had lost a selective property in process of being doubly transformed. These percentages are sufficiently close to the 20-25% doubles recognized in the forward transformation, *ms* → *MS*, to suggest that there is no serious inconsistency in the picture outlined. It will be noted from both tables 1 and 2 that linkage of penicillin resistance, *P*, with

TABLE 2  
*Linked transformations involving loss of a selective property*

CHARACTERS PRESENT IN		PROPERTIES FOUND IN SINGLE COLONY TRANSFORMANTS		
Donor DNA	Recipient cells	Selected strains	Indicated constitution	
<i>Ms</i>	<i>mS</i>	145 <i>M</i> 60 <i>m</i>	124 <i>MS</i> 60 <i>mS</i>	21 <i>Ms</i> 0 <i>ms</i>
<i>mS</i>	<i>Ms</i>	134 <i>S</i>	118 <i>MS</i>	16 <i>mS</i>
<i>sP</i>	<i>Sp</i>	70 <i>P</i>	70 <i>SP</i>	0 <i>sP</i>

Symbols as in table 1. The italicized symbols represent the determinants introduced from the DNA. (Experiments with J. Marmur and D. Lane.)

*M* or *S* could not be detected. Also, the *S* marker was not eliminated from any of sixty cells exposed to *Ms* DNA but not acquiring the *M* property; a smaller frequency of replacement of *S* by *s* in this unselected part of the population is not excluded, and in fact it probably occurs, but is difficult to detect. There is increasing evidence, not presented here, that sulfanilamide resistance will ultimately prove to be loosely linked to streptomycin resistance.

It is concluded, therefore, that definite loci for the allelic gene pairs, *M,m* and *S,s*, exist in *Pneumococcus*, and that these loci occur in linked association within single particles of the cellular DNA.

## TIME COURSE OF TRANSFORMATION

The over-all characteristics of the transformation processes can be determined conveniently with use of the quantitative resistance markers. Drug resistance can be recognized very sharply as appearing 30 minutes or so after the addition of DNA. The marked DNA can be added at a controlled time, and at a known time afterward, deoxyribonuclease, which will destroy all further activity of the free DNA that is present and allow exact knowledge of when the DNA is reacting with the competent cells. About 30 minutes afterward, when the total population is growing more or less exponentially, there is a very sudden development in the phenotype, of the drug-resistant property, far above anything that is going on in cell division, then a delay period, a lag, in which there is no further increase in resistant cells until they begin to divide normally two or more hours later.

Here may be apparent the signs of separation between the two functions of genes; the induction of the phenotypic response which occurs at a time probably determined by the metabolic exigencies of the cell, followed by a delay period, and then eventually an assumption of the other role of the gene, that of producing more cells with more genic material. There are eventually established resistant clones of cells from this process; and they later come to grow in parallel with the total population. As yet these processes cannot be separated biochemically from bacterial division, and the cells must still be growing during both the period of development of the phenotype and that of replication of the genotype.

The nature of the lag, or plateau, of the transformation curve is of interest. There would be two main possibilities: it might be that the transformant cells do not divide for a time, being simply retarded because of having accepted foreign DNA. Instead of a division lag, there might be a segregation type of lag during which the new cell, although drug resistant, has not learned to incorporate in both its daughter cells at division the newly introduced genic material.

There is an experimental way to study this question. If the population is spread out on agar plates just after the DNA has been added, a cell in division lag will eventually give a pure clone of drug-resistant cells. A cell in segregation lag will give a mixed clone, with both sensitive and drug-resistant cells in it.

After replica plating, to detect resistants, the original colony may be examined to see whether or not it is a mixed colony. Mixed colonies are found containing many sensitive cells which cannot survive the drug. There are questions arising here about the purity of the clones, but by allowing appropriate time for separation of any diplococci that might be present and so on, it seems at present safely established that the transformant cells do produce mixed clones for several divisions after DNA has been added to them. We are engaged at present in a study of the distribution of linked characters, the *M* and the *S*, in these populations as compared to the distribution in the whole population.

Transformation can therefore be looked upon as the invasion of the cell by a DNA arising in another cell with different genetic potentialities, the rapid institution of phenotypic response caused by the new material, and a slower incorporation of this foreign material into the genome of the invaded cell. The relations of the competing agents, one displacing the other and so on, seem to indicate that very few genetically effective particles reach the competent sites in any transformation experiment. There may be many DNA particles reacting with the cell surface, but very few have an opportunity to exchange material with the preexisting genic material.

#### DEPENDENCE OF TRANSFORMABILITY ON CELLULAR STATE

Further ideas on the nature of the transformation derive from experiments relating to the state of the cell at the time when it is most susceptible to transformation. It may be recalled that one DNA can interfere with the response to another DNA. This can be a very dramatic experiment in which a DNA preparation having one set of markers can be

added as little as 5 or 10 seconds before another DNA—whence it will be found that most of the cells have already irreversibly responded to the first added DNA, and those which remain are for a time untransformable by the second added DNA.

A little later, new cells are found which are able to respond to the second added DNA (Hotchkiss, '54). At the later periods the new cells which have become transformable are inhibited only partially; the cells now have access to both these DNA's in a certain concentration ratio, and the ratio will determine the amount of inhibition. Here is an indication that new cells are continually appearing in the population, able to respond to DNA; one can block off any particular set of them, adding the inhibitor in advance, and more or less obliterate the response of the cells present at that time, then observe the appearance of new ones as time develops.

Other indications that this is happening in the population are seen in response to the streptomycin-resistance factor, based on the quantitative assay through successive overlapping periods. These assays indicate groups in the population which respond for a time and then cease to respond, while similar new groups are continually appearing in each successive time interval (Hotchkiss, '54). This immediately suggested a relation to the cell division cycle. The falling off of these groups occurs between 10 and 20 minutes, well within a division cycle of 30 minutes. It seemed possible that if one could do anything to modify the cell division cycle, one might be able to modify the response to the transforming factors.

It seemed that the processes leading to cell division, like other biochemical processes, must be in a steady state, conditioned by various enzymes. A steady state would mean that each enzyme in a series would be turning over as many micromoles of substrate per second as it received from the preceding system. Such a state, achieved at a temperature of 37°C., would not necessarily be a steady state for some other temperature; if the culture were cooled to another temperature, say 25°, the system would be thrown out of balance.



Some systems would be less affected than others, so there would be piling up of substrate at certain stages. If there were piling up in a process that led to cell division, a degree of synchronization might have been achieved even in a population of millions of cells.

If a culture established at 37°C., is held for 10 minutes at 25°C. (at which temperature some metabolic processes still go on) then returned to 37°, something has happened so that processes which formerly were continuous are now discontinuous (Hotchkiss, '54). Without such treatment cell division in such a population is going on more or less exponentially with time. Immediately after such a cooling conditioning period there are found at 37°C. waves of rapid, and then slow, cell division as measured by colony count. What would otherwise be a linear growth curve is broken up into repeating cycles, having an over-all time of 30 minutes and a logarithmic increment of 2. One-half of the divisions occur during 4 minutes within each cycle of 30 minutes. The simplest interpretation is that a considerable degree of synchronization of bacterial cell division has been achieved, although to explore that area of cell physiology would be a study in itself.

The point concerned here is that, after this same temperature-conditioning pretreatment, a rather remarkable "phasing" or cycling of transformability is also obtained. During the period at the lower temperature there is an increase in the number of cells which are transformable; but soon after they are back at 37°C. — the time when divisions occur rapidly — there is a drop to negligible transformability, then a rise again. There are normally several successive rises and falls after a single pretreatment, so that a true cyclical process has been induced, rather ruling out the possibility that one has simply modified some decay process and added it to a rising process.

These cycles of transformability eventually become out of phase with what seems to be the average division cycle for the rest of the bacteria. In transformability, atypical cells may be involved, or at least additional variables. But in any case

those environmental factors which have apparently partially synchronized the division cycles have modified in a cyclical fashion also the transformability of large populations.

#### SUMMARY

It appears that in bacterial transformations one is introducing gene material in the form of DNA particles which contain negligible amounts of protein, if any. In some cases these DNA particles exhibit what appears to be a typical genetic linkage between phenotypically unrelated characters. The incorporation of this genic material from another line into the recipient cell occurs in two steps. The added DNA introduces its own enzymatic or phenotypic response within a few minutes, and only later comes to be fully incorporated into the existing genome of the recipient cell. During this time, there is a kind of exchange process in which determinants of the recipient cell are eliminated or displaced, a mechanism reminiscent of crossing over, controlling which part of the genetically linked determinants of the incoming DNA will ultimately be retained by the cell. The ability of the recipient cell to respond is in some way dependent on the stage of the division cycle which it has attained. Thus the bacterial transformations are focusing attention on the critical genetic processes that go on in cell division, probably no less important than the events of sexual cell fusion, already so well documented.

#### DISCUSSION

CASPARI: Did I understand you to say that the number of cells which are obtained in this state, where they can be influenced by DNA, is one of the limiting factors on the number of cells that can be transformed? Is it the only point besides concentration of DNA?

HOTCHKISS: The concentration response to amounts of DNA gives a curve which rises linearly then approaches a saturation level. That tends to indicate two things. One is that the reaction is probably in the nature of a single-hit

type of interaction; and its reaching a kind of plateau indicates that there are a limited number of competent bacterial sites. We have had as many as 17% of the cells susceptible. However, strain differences, the nature of the medium, and the presence or absence of serum albumin all modify the yield.

LINDEGREN: Dr. Emerson once suggested that the surface of the chromosome is a mirror image of the substrate, and it seems to me that there are increasing pieces of evidence which indicate that it is a reasonable point of view. DNA *as such* may not be the inducing agent but the conformation at the site of the chromosome where the gene is located may be the agent which induces the character.

I think our recent evidence that galactose is itself a mutagenic agent for inducing the capacity to ferment galactose is a case in point. Additional support for this view comes from a study of a locus which we call *MZ*. This gene responds to five different inductors with the production of a specific adaptive enzyme — maltose, turanose, sucrose,  $\alpha$ -methyl glucoside, and melezitose. The gene exists in a series of multiple alleles; the characteristic of the multiple alleles is the loss of the specific ability to respond to certain inductors. One allele will not respond to sucrose,  $\alpha$ -methyl glucoside, or melezitose. Cultures carrying this allele, when presented with a mixture of maltose plus sucrose cannot produce the enzyme, indicating that sucrose can block the action of the gene in the production of the enzyme.

These pieces of information support the idea that what happens at the surface of the chromosome is not due to the DNA as such but to a structural formation which DNA incidentally preserves and which may be transferred in some way to the offspring by the induction technique.

HOTCHKISS: The only thing I might say is if this is a process where DNA affects something which affects the enzyme, there is no particular conflict in the possibility, as found with mannitol actually, that the substrate might also induce a part of this process. I think Dr. Lindegren is really asking whether there may not be a reversible relation between some "actual"

gene and the specific DNA, either one of which can initiate the gene effects. I have no particular feeling or objection about that. We still have to consider the gene as operationally replaceable by DNA.

PLOUGH: Is that quite the idea, Dr. Hotchkiss? As I understand Dr. Lindegren, he is suggesting that this is a nonspecific effect rather than a reversible effect.

HOTCHKISS: Perhaps that is what Dr. Lindegren meant. However, then we certainly must testify that there is high specificity in the relation of the source of the DNA, the properties of the clone or strain, and those which it can induce in the transformation.

EHRET: I wonder if you would comment on alternative interpretations instead of the classical genic interpretation — perhaps the sort of thing recognized by Lederberg in his review under the general heading of hereditary symbiosis? The principal objection to such an alternative in which a temperate phage particle may be causing the phenomenon in question seems to be the necessary protein content. Now what would even as little as 0.01% of protein in these DNA extracts represent in terms of phages and in numbers of bacteria transformable? And a second point against the alternatives is the evidence of linkage. But might we have something analogous to linkage, that is, nonindependent assortment, by a nearly simultaneous multiplicity infection of heterogeneous particles, with some probability of mutual exclusion?

HOTCHKISS: We have answered the last part by using different mixed DNA's having the separate properties. We not only tried the mixture, but grew two kinds of cells — the *M* cells and *S* cells together — so we could precipitate the fibrous DNA particles, thinking to stick them together, but were not able to do so in that way. We feel that the frequency data indicate that successive single transformations did not account for the doubly marked cells.

As for the low protein content, of course all we are saying is there could be as much as 0.02% of protein, since our methods cannot possibly go farther than that. Protein would be as good



a candidate for a gene as DNA, and if present would not give particular help in relating it to bacteriophage or bacteria. I think Dr. Zinder will more or less agree that it is a better explanation of transduction to think of it as a modified transformation, where bacteriophage is a carrier, than to think of the more efficient transformations that we have with DNA — in which we cannot detect either phage, bacteria, or proteins — as being explained by something itself not fully explained.

EHRET: Does caffeine have any effect? Do the other bacterial DNA's have any effect in inhibiting?

HOTCHKISS: We have seen no effect of purines. I indicated that pneumococcus DNA having one marker will inhibit others. And in trying to make a species transfer from streptococcus to pneumococcus, the very streptococcus DNA which should have borne streptomycin resistance is actually inhibitory to the streptomycin-resistance transformation by pneumococcus DNA.

STENT: Does the presence of DNA have any adverse effect on the bacteria as such? Do you observe any deaths?

HOTCHKISS: We noticed the lag in replication of cells with the transformant phenotype. We thought perhaps there would be a severe setback response to any kind of DNA, including the unmarked DNA particles which are being absorbed. There does not seem to be; in fact, the observed lag seems to be a segregation lag.

BILLEN: Do you have any idea of the size of the effective particles that might penetrate the cell, or of the actual penetration?

HOTCHKISS: Not really, at the moment. The particle size is believed to be a few million in the typical DNA.

MILKMAN: How much variability is there in segregation lag?

HOTCHKISS: It is quite variable. The mean of the broad distribution would be somewhere in the range of about four to five divisions. Of course we cannot determine the extremes accurately.

SKAAR: In constructing your regression of the amount of transformation in time, is your initial burst measured by plating directly on streptomycin—implying that resistance is dominant over sensitivity?

HOTCHKISS: It is essentially that. The original modified cell seems to be a resistant.

COOPER: I wish to question the now widely accepted conclusion that DNA can be taken to be the genetic material. The conclusion that DNA, itself, is the transforming principle is based chiefly on the purity of Dr. Hotchkiss' preparations of transforming principle. Yet Dr. Hotchkiss states that his preparations may contain as much as 0.02% protein.

Regrettably, Avogadro's number is a large one, and a conservative calculation suggests that Dr. Hotchkiss' purest preparations could contain somewhere between 1 and 10 million protein molecules per microgram. This being the case, the evidence at hand is compatible with *at least* three interpretations, and a choice of any one of these is not dictated by any facts or experiments known to me. These three possibilities are: transforming principle is DNA, transforming principle is protein, and transforming principle is a complex of protein and DNA. No one of these possibilities is incompatible with any results so far obtained. If protein were protected by DNA, and in turn is the transforming principle or a necessary component of it, then of course destruction of DNA by DNase will inactivate the preparation. Conversely, owing to DNA protection, the preparation will remain active even if subjected to proteinase.

Assuredly, the rarity of the event of transformation with DNA preparations suggests, *among other possibilities*, that a molecule relatively rare in the preparation is the transforming principle. Again such a molecule might be protein, a protein-nucleic acid complex, or indeed a special type of nucleic acid that is an uncommon component of the whole preparation.

In principle, the possibility that protein is an essential component of transforming principle, or is itself transforming

principle, would seem testable. If the nucleic acid, which is alleged to be present in at least  $5 \times 10^3$  times the concentration of the protein, is the transforming principle, and the protein not, then the use of one sample over and over again might result in relatively little loss of transforming activity. If, on the other hand, protein is an essential component, the preparations should show exhaustion effects with repeated use. Again, it may be possible to test this notion by radiation inactivation experiments.

Perhaps the cytologist has not often enough emphasized some of his observations, or some of the uncertainties connected with his interpretations. In this connection, I should like to emphasize one "fact," and that is the *negative* correlation between nucleic acid "charge" and genetic activity in chromosomes. The genetically inert regions of genetically active chromosomes, as well as genetically inert chromosomes themselves, are positively and densely heterochromatic (i.e., especially rich in nucleic acids), whereas the genetically active regions may appear very lightly staining with Feulgen, orcein, and so on, or they may absorb ultraviolet much more lightly. Secondly, it is odd that the chromonemata appear packaged in nucleic acid when the nuclear membrane is gone and the chromosomes of a dividing cell are in the presence of the cytoplasmic substrates. Is the entire envelopment of the mitotic chromosome within its nucleic acid a protective measure necessary in the absence of the selectively permeable nuclear membrane?

Lastly, an uncertainty that ought to be emphasized may be mentioned. We really do not know whether genes in salivary gland-type chromosomes are in the dark-staining, nucleic acid-rich bands, in the light interbands, or in both. On analogy with the genetic inertness of the heterochromatic regions of these very chromosomes, we may suspect that the dark bands—far from being genic—are spacers of inert material, so to speak, and that the genes lie in the relatively nucleic acid-poor interband. Condensation of such chromosomal structure may involve the enclosure of the genetic material within the

supposed genetically inert, nucleic acid-rich protective spacers to each side.

None of the experiments or facts, from the very beautiful biochemical research on transforming principle to the possibly equally informative work of cytologists and geneticists, leads directly and unambiguously to the conclusion that transforming material or genes are nucleic acids, or largely composed of nucleic acids. I would appreciate learning whether or not the decision, so widespread today, that nucleic acid is a transforming principle has in fact been decided by an unequivocal experiment, or whether it is no more than a voted agreement at the present time.

HOTCHKISS: This is not the kind of question for which an absolute answer or experiment can be offered. I would say that the same argument, of course, still can be applied as to whether enzymes are proteins. It has never been shown that there are not small amounts of unknown, undetected materials in enzymes that go along with the crystallizable proteins and are inactivated when the protein is damaged.

Even though we have more or less come to believe (perhaps by voting agreement) that most of the enzymes are proteins, we still do not think that all proteins must be enzymes. In this same way, one need not suppose that all DNA must be genic. Therefore, it is not disturbing to find chromatin for which no function is known, or as we have done, part of our DNA which shows less activity than the remainder.

But if we elect protein as transforming agent, because of the familiar properties of protein — specificity, subtlety of structure and so on, which we now know of course can be expected also in DNA molecules — we must admit that the protein which is being assumed is otherwise a most unusual protein. It is a protein which is active in these very small amounts. It is a protein which is very stable to heat, to all sorts of things — proteolytic enzymes, alcohol, and surface denaturation. At the same time that other proteins which are present are being separated successfully from a pneumo-



coccus preparation, this protein is being concentrated and retained.

Furthermore, one must now add that it is stable to deuteron, electron, and X-ray bombardment; and that the volume and size relation of the vulnerable target roughly indicate molecular weight of the order of millions like the DNA molecules and not as small as those of typical proteins.

But of course one can still say that the DNA is being inactivated and the hypothetical atypical protein is being released. Why is this undetected protein so stable in the presence of DNA but instantly denatured and inactivated when any one of a number of things is done which you know modifies DNA but does not even destroy or remove it? I feel that the demonstration is really up to those who would like to bring evidence that protein is present, and that it is no longer a prime obligation to bring further evidence that protein is absent. I would like to add two things. Dr. Cooper mentioned a type of experiment which we have begun, absorbing biological activity by one culture and testing for the exhaustion of it. Even at a DNA concentration so low that the agent is altogether limiting for transformation, the amount of material absorbed in transforming several thousand cells cannot be detected as a drop in biological activity. We can demonstrate, therefore, that the active material actually utilized is only a small fraction of the activity which is present.

There is another point worth mentioning. Calf thymus DNA is a very potent and successful inhibitor of transformation. It is hard to see how something which is supposed to be the classical prototype of DNA can carry a protein so exquisitely adapted to the pneumococcus that it in turn can inhibit the picking up of this hypothetical protein of the pneumococcus.

SPIEGELMAN: I am rather convinced it is DNA. It must, however, be admitted that the possible involvement of a protein has not been eliminated. I should like to suggest an experiment which might be sufficiently sensitive to provide

an almost definitive result. If cells were grown in reasonably active  $S^{35}$ , the efficiency of removal of protein from the transforming principle as it is purified could be followed to levels not assessable to other methods of analysis.

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# BACTERIAL TRANSDUCTION

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## FOUR FIGURES

The specific genetic consequences of bacterial transformation reactions, as described by Hotchkiss (this symposium), are paralleled in the transductions that have been demonstrated in the bacterial genus *Salmonella* (Zinder and Lederberg, '52; Stocker *et al.*, '53). That is to say, heritable alterations of discrete bacterial characteristics are brought about by the application of specific materials; in transformation by deoxyribonucleates (Avery *et al.*, '44) and in *Salmonella* transduction by bacteriophage-carried material (Zinder and Lederberg, '52; Zinder, '53), probably of similar composition.

## TRANSDUCTION

This report will concern itself primarily with transduction as found in *Salmonella typhimurium*. This organism is almost as well suited for laboratory handling as its "cousin" *Escherichia coli*. It is nonfastidious, grows on simple defined media, and offers a wide variety of markers for genetic analysis.

The general aspects of transduction can be demonstrated by the following experiment. A sterile bacteriophage-produced lysate of a strain (the donor) which was nutritionally independent, fermented the sugars galactose and xylose, and was streptomycin resistant was applied to a strain (the recipient) with the reciprocal characteristics. Any alterations in the recipient were observed by plating large numbers of organisms upon media selective for the characteristics of the donor. Each of the four characteristics was altered in significantly

higher frequency than could be explained by mutation and each independently of the others (see figure 5 of Zinder and Lederberg, '52). The number of transductions for any particular marker was a linear function of the amount of lysate applied. The transductions were not due to the presence of a general mutagen since the donor usage of organisms which were similar to the recipient for any one of the four markers restricted only the transduction of that particular marker.

The transformed cells are stable for the new characteristic and, in fact, will provide a source of this characteristic for subsequent transduction.

A wide variety of differentiating characteristics, both pre-existent and induced, have been transduced within and between species and strains of *Salmonella*. In almost every instance a characteristic for which selective procedures were available has been transducible. To my knowledge, only an occasional mutation to streptomycin resistance has been refractory. Lacking other means for the analysis one can only speculate as to the reason for this. It might be pertinent to point out that extensive tests for complete genetic recombination in *Salmonella typhimurium* have thus far all failed.

The characteristics studied have included nutritional, fermentative, antigenic, morphological, and drug responses. Because the frequency of transduction is usually of the order of one per million treated cells, it is generally possible to transduce in only the direction for which selection can be applied, e.g., nutritional dependence to nutritional independence. However, with mutually exclusive (allelic) antigen factors, Lederberg and Edwards ('53) have been able to transduce in both directions. It is interesting to note that in the transformed cells containing the transduced antigen factor there is no evidence for either the previous phenotype or genotype. Thus transduction involves not only addition of factors but also a substitutive replacement.

Again transduction can not only transfer over bacterial characteristics, but — as in any genetic system — it can dissect similar phenotypes controlled by different genetic factors.



Isolates of mutations causing the same phenotype can often transduce each other to wild type (Zinder and Lederberg, '52). By cross testing of many different nonmotile *Salmonella* strains (cells lacking the locomotor organelle, the flagellum), Stocker and his coworkers ('53) have found at least seven different genes that control flagella formation. Thus transduction provides a test for nonallelism.

In the many instances of transduction cited there have been but few exceptions to the rule that individual markers are transduced independently of the others. Most of the previous studies have been with donor and recipient strains of different origin. In order to have isogenicity, except for the marker in question, a series of mutants have been obtained in a strain which was used both as donor and recipient. Five nutritional and two fermentative markers have been tested for linked transduction with one another and five other nutritional markers. Linkage of nutritional markers is sought in the following way. A lysate is obtained from one mutant requiring, for example, histidine for growth and applied to another mutant requiring, for example, tryptophan. The treated cells are plated upon medium containing the requirement of the donor, histidine. The transductions are then tested for a histidine requirement by replica plating (Lederberg and Lederberg, '52) to minimal and minimal-plus-histidine media. In this manner several hundred transductions can be readily scored. Linkage of nutritional to fermentative markers is sought by direct scoring of the transductions upon a medium which is an indicator of the fermentation. The study is a continuing one, but as yet no linked transductions have been found.

A number of instances of linked transduction have been found (Stocker *et al.*, '53; Kauffmann, '54) in studies of the factors controlling the function and antigenicity of the bacterial organelle, the flagellum. The flagella have associated with them a diagnostic antigen characteristic of the particular *Salmonella* species. The antigen-controlling loci form an allelic series (Lederberg and Edwards, '53).

When a nonmotile strain is transduced by a motile one which has a different flagella antigen, the transductions to motility generally exhibit the inferred flagella antigen of the recipient, not that of the donor. This then provides a separation of the genetic factors controlling antigenicity and function. In a number of instances not only are there found transductions to motility with the unveiling of the flagellar antigen latent in the recipient, but also motile cells with the antigen of the

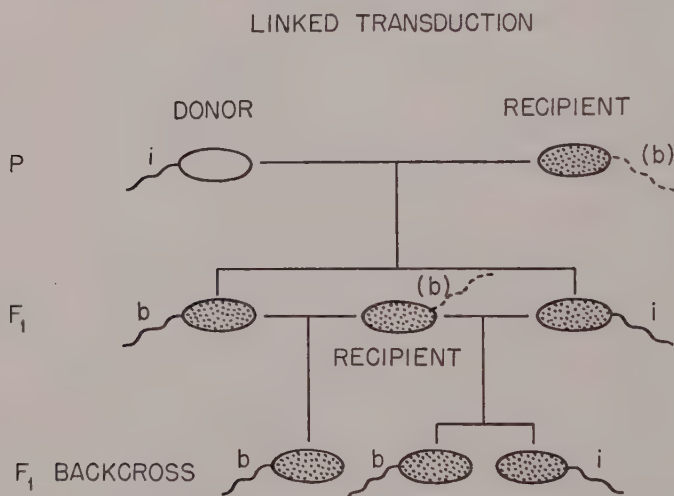


Fig. 1 A schematic representation of one example of linked transduction. The dashed line represents the absence of flagella. The small letters represent the existing or latent flagellar antigen.

donor, and in about equal frequency. Figure 1 is a diagram of one example of such linked transduction. A nonmotile strain which was presumed to be *S. paratyphi* B and, therefore, should have had flagellar antigen *b* was transduced with a lysate from a motile *S. typhimurium* strain with flagellar antigen *i*. Motile cells were obtained, some with antigen *b* and some with antigen *i*. These latter assumedly received both a factor for motility and one for antigenicity. The linkage can be verified by progeny testing of the transductions. Lysates prepared from the *b* cells and applied to the original recipient

give only motile *b* cells, while lysates from the *i* cells give both *b* and *i* cells. A number of similar instances of linkage of different flagellar loci and the flagellar antigen locus have been found. By appropriate cross testing, Stocker (personal communication) has been able to position (map) three flagellar factors with respect to the antigen locus.

Transduction then seems to involve the following: During the lysis of the donor bacteria active genetic material is released which, at least in some instances, materially encompasses more than one gene and, taken as a whole, encompasses the entire bacterial genome. If the bacterial genetic material is organized into chromosomes, then some degree of fragmentation must have occurred. It is not possible at this time to estimate the size of such fragments, but it would not require a very high degree of fragmentation to separate the numbers of markers that have been tested. For example, ten markers distributed at random among the chromosomes would with as few as forty equal-sized fragments exhibit no linkage. These fragments, when applied to appropriate recipient bacteria, can replace their genetic homologs and thereafter be retained as an integral part of the bacterial genome.

#### TRANSDUCTION AND BACTERIOPHAGE

It was previously stated that the transduction was mediated by bacteriophages. The rationale behind this statement will be taken up later, but it is necessary first to digress for a discussion of the bacteriophage and its life cycle.

Luria ('53) defines viruses as "... submicroscopic entities, capable of being introduced into specific living cells and of reproducing inside such cells only." The bacteriophages are those viruses attacking bacteria. Virulent phages, upon infecting appropriate bacteria, reproduce in every cell and ultimately lyse the cell releasing the phage progeny. Infected cells never survive the infection, even though phage reproduction may be inhibited; that is, nonviable phage will kill the infected cell. Temperate phages, on the other hand, exhibit two alternative pathways subsequent to infection (Lwoff, '53); one

paralleling the action of virulent phage and the other establishing with the infected bacterium a symbiotic relation (lysogeny). The proportion of cells in any culture reacting in either manner may be altered by the physiological environment. It is quite apparent that transducing phages must be in this latter category. However, much of what is known about phage reproduction has been ascertained with the virulent phages and we shall use them as a model, modifying the detail as necessary when the information is available.

After the mixing of a phage and its host the phage disappears for some time (latent period), and then the culture lyses and phage is again found in the supernatants. The events occurring during the latent period can be further studied by prematurely lysing the cells by a variety of means (Doermann, '52). Again there is a period, about half of the latent period, in which no infective phage is found, and then phage accumulates continually up to and through the time of the beginning of spontaneous lysis.

Further fractionation of the latent period has been accomplished by the use of chemical and radioisotope procedures. The bacteriophages are composed of almost equal parts of deoxyribonucleic acid (DNA) and protein. Hershey and Chase ('52) have shown for coliphage T2 that the nucleic acid is probably responsible for the genetic continuity of the phage. The phage T2 has a pyrimidine base, different from its bacterial host, hydroxymethylcytosine in place of cytosine (Wyatt and Cohen, '52). By following the ratio of the bacterial pyrimidine to phage pyrimidine subsequent to infection, Hershey and coworkers ('53) have shown that the appearance of mature infective phage is preceded by the growth of a phage DNA precursor (vegetative phage), some of the component parts of which come from the bacterial host, the rest from new DNA synthesis. After a period, samples of the vegetative phage are matured by acquiring phage protein, presumably as a surrounding coat. This latter is responsible for the phage's adsorptive and antigenic properties.



The temperate phages, when in the lytic cycle, apparently undergo similar processes. However, they may fail to initiate vegetative growth and, by as yet incompletely understood mechanisms, establish lysogenicity. The cells become carriers of what has been called prophage which is in essence the carrying of a potentiality to produce, either spontaneously (with a low fixed frequency) or by specific induction procedures, mature phage (Lwoff, '53). Several facts have led to the postulation of an intimate relation between the bacterial genome and the prophage. The general stability of the lysogenic condition (the failure of the prophage to segregate during vegetative growth of the bacteria), the difficulty and rarity with which multiple lysogenesis for mutants of the same phage can be established (Bertani, '53), and the segregation of lysogenesis in crosses of sensitive by lysogenic (Lederberg and Lederberg, '53), all support the notion of a chromosomal site for the prophage. Lysogenic cells exhibit some properties differentiating them from their sensitive parents, in particular, they are immune to the application of the same or related phage.

The phage with which we shall be concerned (PLT-22) was obtained from a lysogenic strain of *Salmonella typhimurium* (Zinder and Lederberg, '52). It can be readily handled with those techniques developed for the virulent bacteriophages (Adams, '50). Some of the phage's properties are listed in table 1. These were obtained by use of a virulent mutant of the phage to facilitate technical problems.

The phage was implicated in transduction by a variety of lines of evidence. Some of the evidence is listed in table 2. It is apparent that the phage as measured by plaque production and by the genetic effects share a host of common properties, probably related to gross morphological structures or the ability of the phage to adsorb to bacteria subsequent to treatment. The limits of possible transduction with this phage are then defined by the phage's adsorptive capacities. However, other phages with other adsorption spectra may be

available. Baron ('53) has reported transduction with a totally unrelated *Salmonella* phage.

The role played by the phage is primarily passive. It acts as a vehicle for the genetic material. The phage may have its specific site on a bacterial chromosome, but its transducing potentialities are in no obvious way limited by its presumed position; all markers are transducible.

TABLE 1  
*Some properties of Salmonella phage PLT-22*

PROPERTY	MEASURE
Size	50-60 $m\mu$ (electron microscopy)
Shape	Hexagonal head with short stubby tail
Adsorption	Rapid
Latent period	30-32 minutes
First mature phage	20 minutes
Burst size broth	800-1000
Plaques	Large and rapid forming
Base composition <sup>a</sup>	Adenine, thymine, guanine, and cytosine (equimolar)
Phosphorus content <sup>a</sup>	10 <sup>-11</sup> $\mu g$ /phage
Phosphorus from host <sup>a</sup>	10-20%
Phosphorus transfer to progeny phage <sup>a</sup>	30-40%

<sup>a</sup> Garen and Zinder, unpublished.

TABLE 2  
*Some properties of the bacteriophage and the transducing material*

PROPERTY	COMMON	REMARKS
Size	+	Ultrafiltration and ultracentrifugation
Antigen	+	Kinetics of inactivation by antiserum
Heat sensitivity	+	Kinetics of inactivation
Site of adsorption	+	Adsorption on a variety of <i>Salmonella</i> species
Osmotic shock	+	Both resistant
Ultrasonics	+	Kinetics of inactivation
Effect of DNAase	+	No effect
Effect of RNAase	+	No effect
Effect of trypsin	+	No effect
Ultraviolet	—	Phage and transducing material with different kinetics of inactivation

Donor cells must of course be phage sensitive. However, recipient cells may also be phage sensitive. The transductions are then found among the lysogenic survivors of the infection, which under certain conditions of multiple infection amount to a considerable proportion of the infected cells (Boyd, '51). Transduction can also be assayed with lysogenic cells, cells already carrying a prophage. In this instance, phage penetrates but does not lyse the cells, neither does it establish lysogenicity nor replace the preexistent prophage. The phage also can transduce cells for which it is poorly adapted for growth,

TABLE 3

*A comparison of the lysogenization of randomly infected cells and the transductions*

MULTIPLICITY OF INFECTION		NUMBER OF TRANSDUCTIONS PER $2 \times 10^8$ BACTERIA	FRACTION OF CELLS AT LARGE LYSGENIZED AND PHAGE	FRACTION OF TRANSDUCTIONS LYSGENIZED AND PHAGE <sup>c</sup>
Phage A <sup>a</sup>	Phage B <sup>b</sup>			
1	0	580	13/40 A	38/40 A
6	0	3900	20/20 A	20/20 A
1	5	670	4/30 A; 26/30 B	2/30 A; 28/30 B

<sup>a</sup> Transducing phage with haloed plaque.

<sup>b</sup> Nontransducing phage with nonhaloed plaque.

<sup>c</sup> Following purification.

lysing but  $10^{-5}$  or less. The frequency of transduction for any particular marker may vary with the differing conditions of the recipient, but by a smaller factor than the phage's lysogenizing capacity.

The following experiment demonstrates explicitly the carrier role of the phage. The recipient cells were phage sensitive. They were infected with one phage particle per bacterium, and the lysogenization of the cells at large and the transductions in particular determined. At this multiplicity some 40% of the cells are uninfected. Of the remaining 60% of the cells about half are lysed, and the other half are lysogenized. We note that a few of the transductions are not lysogenic (table 3). Another aliquot of the cells were infected with six particles, and we note that there is no evidence for interference, more than one particle can transduce. Still

another aliquot was infected with one effective phage particle and five particles which could not transduce the marker in question. The secondary phage could be differentiated from the transducing phage; it was a plaque morphology mutant. The lysogenizations of the cells at random and of the transductions were the same. The transductions were lysogenized in accordance with the ratio of the two phages, not according to the phages transducing abilities.

The phage, although essentially passive, can in some instances markedly affect the efficiency of transduction. With sensitive cells, the phage lysogenizes some proportion of the cells, and it is among these that transductions are found. Both the physiology of the cell and the number of infecting phage particles, in this instance, affect the frequency of lysogenization. In infection on the other kinds of recipient cells there is an as yet barely known phenomenology, and the line between phage-determined and transducing effects may not always be clear. We may cite one example where the kind of recipient, cell physiology, and multiplicity of infection all play a role. When phage-sensitive cells which have been lysogenized (carry prophage and are immune to superinfection) are used to assay transduction, the efficiency of the transduction falls about a factor of 20. If the cells are old, the assay is not quite linear with the multiplicity of infection. With a multiplicity of one, there is obtained one-third to one-fourth of the number expected from a multiplicity of five. If, on the other hand, young log-phase cells are used, almost no transductions are found at a multiplicity of one. That this is not an adsorption artifact is shown by the restoration of transductions upon addition of about twenty nontransducing phage particles. In all these instances, all the cells survive the infection. Thus, dependent on the physiology of the cells, the concerted action of a number of extraneous phage particles are required to bring about transduction.

If the phage is considered to act primarily as a carrier, the proposed mechanism of phage infection, i.e., injection of nucleic acid (Hershey and Chase, '52), makes it seem likely



that transduction combines the features of phage infection, the injection of phage nucleic acid, and bacterial transformation the penetration and establishment of unaltered bacterial nucleic acid. Some attempts to elucidate the mechanism whereby the bacterial and the phage nucleic acid come together will now be discussed.

Since the wild-type temperate phage lyses only a fraction of the infected cells, the following experiments were carried out with a virulent mutant of the phage—one unable to establish lysogenicity and lysing almost all of infected sensitive cells. This virulent mutant does, however, transduce in the proper assay system at frequencies comparable to those of the parental phage. There are two techniques for assaying transduction by virulent phage: (1) use of cells that are lysogenic, immune to lysis by this phage although it does penetrate, and applying to a constant number of cells some dilution of phage; (2) use of phage-sensitive cells, infected at a multiplicity of one or less with the virulent phage to be assayed, and simultaneously infecting them with a high multiplicity of nontransducing, lysogenizing phage. With this procedure, over 90% of the cells survive the infection. The disadvantage of the first procedure (fall in efficiency and nonlinearity of the assay) has already been mentioned. The second procedure is limited in that the ratio of effective phage to cells must be kept low. The data presented were collected by both methods and so will not always have the same absolute value.

The concept of nontransducing phage has been introduced and its uses pointed out. It was necessary to determine whether there was any carryover at all of transducing material from phage generation to phage generation. Phage was recovered from a histidine-independent ( $H^+$ ) strain and found to contain three histidine-transducing elements per million phage particles; a transduction/phage ratio (T/P) of  $3/10^6$ . From this preparation,  $5 \times 10^8$  phage containing 1500  $H^+$ -transducing elements, were used to infect  $2.5 \times 10^8$  histidine-dependent cells ( $H^-$ ). The yield of phage was

$7.5 \times 10^{10}$ . If we assume 100% transfer of the bacterial genetic material, the  $1500 \text{ H}^+$  elements should have been distributed among these phages giving a T/P of  $2/10^8$ . The activity found was less than  $1/10^9$ . Thus there is little if any transfer of transducing material from first to second generation phage, despite the fact that about 40% of bacteriophage phosphorus (table 2), presumably reflecting nucleic acid, is transferred. Therefore, all transducing elements associated with any phage preparation were obtained during the previous growth cycle.

Most of the studies on the incorporation of bacterial elements into phage have been concerned with a histidine marker. Histidine-independent cells are prematurely lysed following infection by the addition of KCN up to  $0.01 \text{ M}$ . Because of the relatively low activity per phage it is necessary to accomplish the experiments at fairly high cell densities. This causes some nonreproducibility in the timing of the experiments, making for some difficulty in comparing experiments, and also causes some loss of phage subsequent to the onset of natural lysis. However, the datum activity per phage (T/P) should be independent of the total amount of recovered phage. Figure 2 shows that for cells grown and lysed in broth the T/P rises rapidly at first, at a rate comparable to that of the phage. In synthetic medium the shape of the curve is similar, but the T/P for any particular burst size is higher in this medium than in the broth. When synthetic-grown cells are lysed in broth medium, the T/P remains constant throughout the experiment, although the phage has a growth curve similar to that of the synthetic system. As determined by end-point data, such things as medium of growth, medium of lysis, and temperature, all participate in determining the amount of material incorporated into phage. Table 4 illustrates typical end-point data and the corresponding burst sizes for the histidine marker.

End-point data have shown that different bacterial characteristics are transduced at different frequencies by the same lysate. But end-point data are arbitrarily fixed by the

moment of lysis of the cells. Figure 3 illustrates the results obtained for three different markers for broth-grown cells lysed in broth. The histidine marker is depicted as following its usual ascending T/P, whereas the T/P for the purine marker remains essentially constant. The leucine marker, which is usually transduced at low frequency, begins to appear just a few minutes prior to the termination of the experiment.

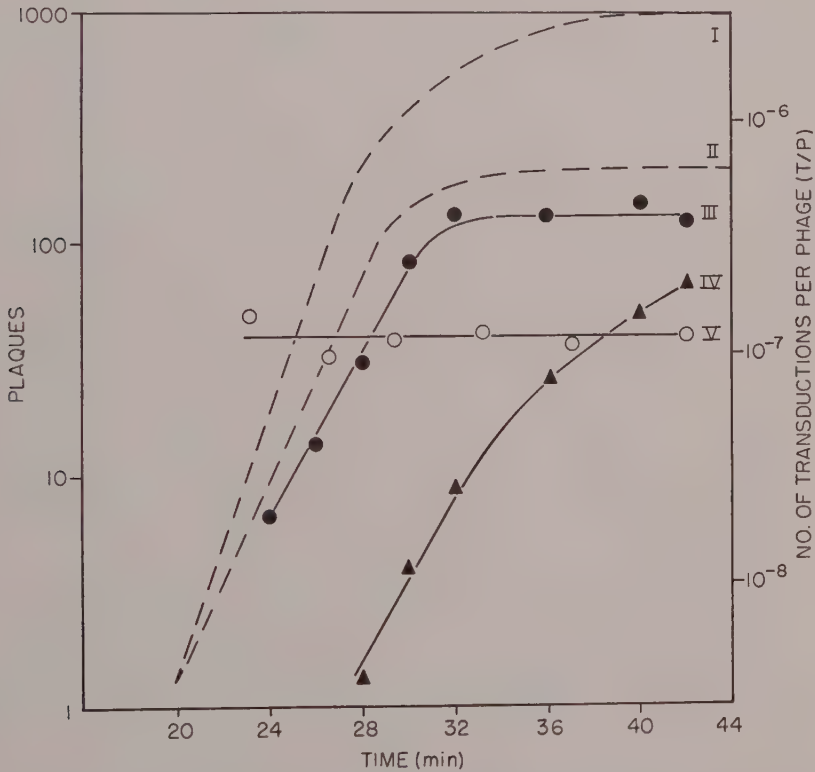


Fig. 2 The growth of phage in different media and its effect on the incorporation of the histidine marker. Curves I and II are idealized growth curves of phage in broth and synthetic media, respectively. The curve for the synthetic media was moved back 2 minutes in time so that direct comparison of the activities per burst size are possible. Curve III represents the T/P for cells grown and lysed in synthetic media, curve IV the same for broth-grown and -lysed cells, and curve V for cells grown in synthetic medium and lysed in broth.

Several other markers have been similarly tested, and it would seem that there are a family of curves bounded at one end by the appearance of mature phage, and at the other by the termination of cell lysis. Within this range, some of the markers appear at a maximum T/P and retain it, others ascend and attain their maximum by the end, and still others are just ascending when the experiment terminates. The end-point T/P for these last markers is the most susceptible to physiological conditions, since anything which would shift the time constants slightly would markedly affect the end-point ratio. In table 4 are compared end-point data for the

TABLE 4

*Effect of environmental conditions on the incorporation of the histidine marker into bacteriophage (end-point data)*

MEDIUM OF CELL GROWTH	MEDIUM OF LYSIS	TEMPERATURE (°C.)	T/P <sup>a</sup>	BURST SIZE
Broth	Broth	37	2.6/10 <sup>7</sup>	1000
Broth	Broth	25	1.0/10 <sup>8</sup>	600
Broth	Synthetic	37	1.7/10 <sup>7</sup>	100
Broth	Synthetic + eas. hyd.	37	9.1/10 <sup>7</sup>	100
Synthetic	Synthetic	37	5.2/10 <sup>7</sup>	400
Synthetic	Broth	37	1.1/10 <sup>7</sup>	450

<sup>a</sup> Assayed by procedure 1.

three and one other low-frequency marker for broth and synthetic medium systems. The purine and the histidine markers show a rise in the T/P, but of much smaller magnitude than the two low-frequency markers. The kinetic data (fig. 4) show a general shift of the curves toward the beginning of the latent period. It may be seen that the total amount of transducing material obtained per bacterium lysed is about the same in both media for the markers that had attained maximum T/P, the difference in T/P being compensated by a difference in burst size. For the other markers, substantial increases in the amount of transducing material were obtained.



In this connection it is interesting to note that in no instance has there been a fall in the T/P as the phage grows. That is, all increments of phage have resulted in an increment in the total amount of transducing material. The phage has not, in the interval allowed before lysis, taken up all of the

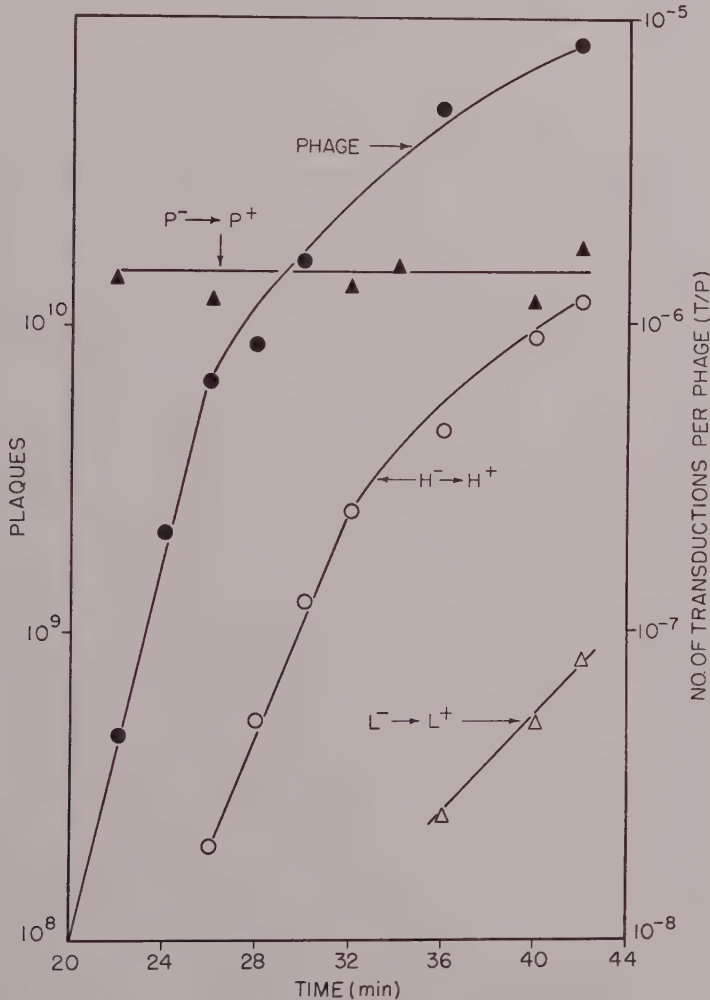


Fig. 3 The phage growth curve from  $10^8$  cells grown and lysed in broth and the corresponding T/P for three different markers—purine (P), histidine (H), and leucine (L).

potentially available material. Although lysis inhibition can be brought about, there is no increase in burst size. The large natural burst size is probably maximal.

We, therefore, cannot extend the boundary at the termination of the experiment. We also cannot delve into the period prior to phage maturation, since it requires infective phage

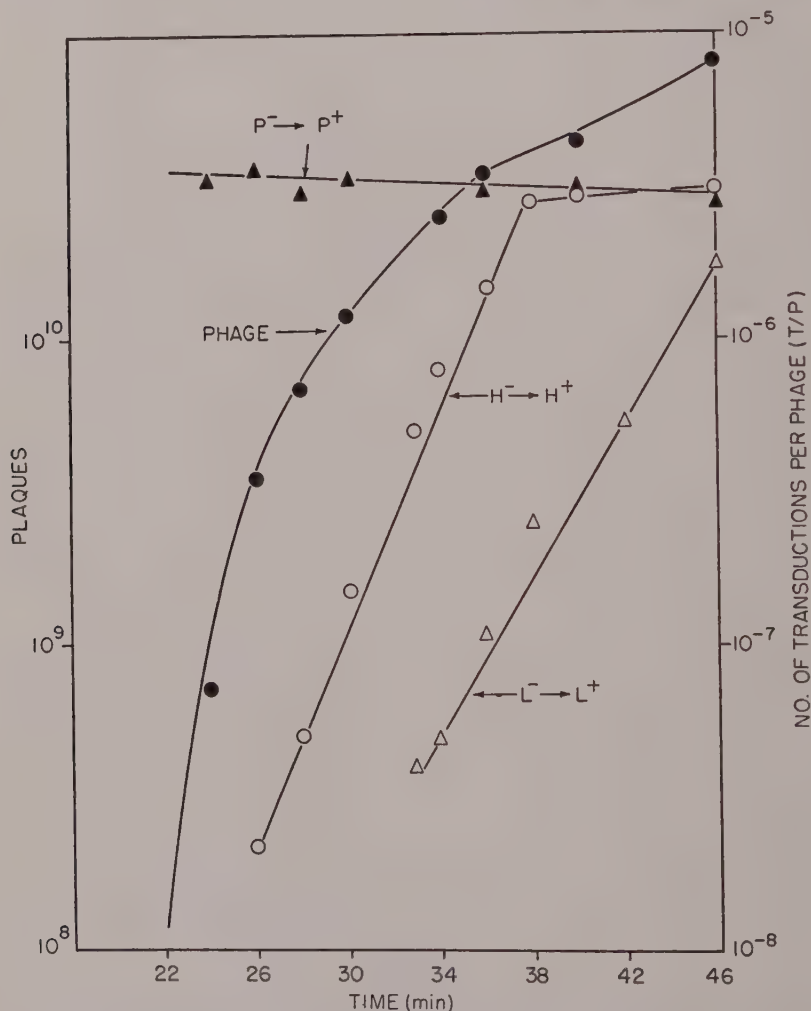


Fig. 4 The phage growth from  $2 \times 10^8$  cells grown and lysed in synthetic medium and the corresponding T/P for three different markers.

for transduction. No transducing particles are found in lysates produced in the presence of the drug proflavin, although presumably here, as with the coliphages, all events prior to maturation occur (De Mars *et al.*, '53). The period subsequent to maturation can be further dissected with respect to phage growth and the incorporation of transducing material. Preliminary experiments with metabolic inhibitors, applied after the beginning of maturation, indicate that, whereas phage growth can be reversibly inhibited, the incorporation of transducing elements is almost irreversibly inhibited. Thus there is no obligate coupling of phage growth and incorporation of active transducing material even in an already functioning system.

#### DISCUSSION

It is quite apparent that transduction as found in *Salmonella* may be considered a transformation, in the pneumococcus sense, aided by having a vehicle for the penetration of bacteria, a bacteriophage. That is, there is incorporated into phage, during its vegetative growth, fragments of the host's genetic material which have retained their biological specificity. The relative stability of this material, especially to the enzyme deoxyribonuclease, may be taken to mean that it is protected by the same structure, the protein coat, that protects the phage nucleic acid. Although this transducing material might be an integral structural entity of the phage, it is unnecessary for the genetic continuity of the phage; it is not heritable.

Once it penetrates the recipient, this transducing material apparently disassociates itself from the phage nucleic acid; the bacterial nucleic acid attempts the transformation and the phage attempts lysogenization, with no obligate correlation of their individual successes and failures. However, the phage may play a role in timing the release of the material transduced, or in altering the receptivity of the cells. We may recall that young lysogenic cells must be multiply infected for transduction to occur, and that this excess phage can be non-

transducing for the marker in question. Experiments by Hotchkiss on the transformability of pneumococci during "synchronous" growth, following heat treatment, suggest cyclical changes in the receptivity of cells for added fragments. It is possible that multiple phage infection could produce similar effects in altering receptivity.

The ultimate mechanism by which added fragments are incorporated into the bacterial genome is unknown. If, as seems likely, the bacterial genetic material is organized into chromosomes, then transduction and transformation both require some kind of crossing-over process between a chromosome and a fragment. For complete integration — these processes then require double crossovers and, consequently, mechanical models of crossing over are complex. A simpler model assumes incorporation of the new material during chromosome replication. The added fragment would pair with its genetic homolog and this complex would act as a template for the duplication.

In viewing the still incomplete data on the incorporation of bacterial elements into phage, we are again faced with complexity. In this instance we cannot, as with coliphage T2, differentiate phage and bacterial nucleic acid. We can only use the model of DNA metabolism, developed for colon bacillus infected with phage T2, by analogy, without being able to confirm it. With T2, after infection, the synthesis of bacterial DNA ceases and the synthesis of phage DNA begins. The pre-existing bacterial nucleic acid is broken down and converted into phage nucleic acid, throughout the growth of the phage (Hershey *et al.*, '53). The *Salmonella* phage obtains almost all of the DNA phosphorus present in the bacterial host, and about half of this phosphorus is transferred to phage progeny (see table 1). The amount of transfer from bacteria to phage seems much too large to represent specifically the transducing material, and the amount of transfer from phage to phage stands in contrast with the complete turnover of the transducing material. The failure of the radioisotope data to correlate with the transducing data may be taken to mean



that the obtaining of transducing elements is only a small part of the over-all reaction, and that here, as in coliphage T2, the major portion of bacterial nucleic acid is converted to phage nucleic acid. If transduction involves the transfer of unaltered bacterial nucleic acid, it must be obtained before it it changed over.

What role does the recipient play in the interpretation of the data? The recipient can influence the absolute value of the assay for any particular marker; that is, determine the probability of the proper particle producing its effect. There is no method of estimating this efficiency factor at this time. The recipient cannot influence the relative values of preparations prepared at different times or under different physiological conditions. The differences in the transduction frequencies of the different markers may be in part due to differences in receptivity, but the fact that the T/P of some markers has become maximal, while others are still increasing, would indicate that receptivity is not the sole explanation.

The following tentative picture for the incorporation of transducing elements into phage is proposed. The maximum amount of material available is a function of the number of infected bacteria. In *Salmonella* cultures growing in the logarithmic phase there are on the average four nuclei per cell, and it is this that we draw upon. It is to be remembered that at the end point the yield is only one transducing particle for any marker per three to five hundred lysed bacteria (although an unknown efficiency factor goes into this calculation), and less per nucleus, and probably still less per gene replica. Thus the data are the averages of the happenings in many individual bacteria which may not be doing the same things at the same time.

The most salient feature of the data is the maintenance of the relative rank of the T/P of the different markers. Three markers *A*, *B*, and *C* which appear among the phage in that order in one experiment, appear consistently in that order in the different physiological conditions. This would indicate that they are not equally available at any particular

time, and this, in turn, may be a reflection of their previous organization. Positioning the genes on some structure, a chromosome which was being broken down in some sequential manner, would be the simplest explanation. The rising T/P could be explained in a similar fashion. That is, as the structure is breaking down and the transducing elements become available, different bacteria reach the same point at different times. What is changing, in time, is not the probability of incorporation, but rather the numbers of bacteria in which incorporation can occur. Since in no instance does the T/P fall, with further phage synthesis, there must be some excess material still available and the amounts depleted by the early incorporation negligible in comparison with the total amounts available.

It is not yet possible to point with any certainty to any stage of the phage growth cycle and say that at this time the transducing elements are incorporated. The data would fit the notion that incorporation occurs at the maturation step, with a constant probability per maturation, but other mechanisms cannot be excluded.

In many ways it would seem that what we obtain in the way of transducing elements are the dregs of reactions more pertinent to phage reproduction. Although temperate phage is requisite for the demonstration of transduction, transduction is not contingent on the phage's temperate character, the ability to lysogenize, but rather seems a reflection of the fact that phage growth involves a kind of nucleic acid parasitism.

#### DISCUSSION

PLOUGH: I have a question based on results of our Amherst group with *Salmonella typhimurium* which bears on the general interpretation of the phenomenon. Transduction is thought of as a series of point-by-point gene changes, and is also described as a series of transformations of the pneumococcus type which may or may not be specific. I should like to present once again the view, based on our data, that a uni-

fying concept is that transductions are the result of non-specific mutagenic effects of phage or phage-released products.

We have one major lysogenic or bacteriophage-carrying strain and a number of nonlysogenic strains. We have reported cases of what appeared to be multiple transductions, but we can no longer maintain this interpretation, since we found that three separate requirements on one strain are determined by one gene.

TABLE 5

*Comparison of end-point data for the transduction of four different bacterial characteristics by bacteriophage grown under different conditions*

MARKER	GROWTH SYSTEM	T/P <sup>a</sup>	BURST SIZE	TOTAL TRANSDUCING MATERIAL PER 10 <sup>8</sup> BACTERIA
Purine	Broth-broth	1.5/10 <sup>8</sup>	800	120,000
	Synthetic-synthetic	3.0/10 <sup>8</sup>	400	120,000
Histidine	Broth-broth	1.2/10 <sup>8</sup>	800	96,000
	Synthetic-synthetic	3.0/10 <sup>8</sup>	400	120,000
Leucine	Broth-broth	8.0/10 <sup>8</sup>	800	6,400
	Synthetic-synthetic	1.7/10 <sup>8</sup>	400	68,000
Serine	Broth-broth	3.1/10 <sup>8</sup>	800	2,400
	Synthetic-synthetic	9.1/10 <sup>7</sup>	400	36,000

<sup>a</sup> Assayed by procedure 2.

We expose one of the mutant strains of nonlysogenic stock requiring the amino acids, histidine, and leucine to a cell-free filtrate from a lysogenic prototroph. We then make heavy surface platings by the ingenious technique of Demerec and Cahn using separate plates supplemented for tests of reversion to histidine-plus and for leucine-plus. Such tests show an increase in frequency in the reversions to histidineless of more than three hundred times, and in the reversions to leucineless of over a hundred times, the spontaneous reversion rate of the controls. If the same strain is subjected to ultraviolet, reversions of both sorts appear with the same order of frequency but at a much lower rate. There are certain differences, however. From counts of the percentage of the mutations which have appeared after successive divisions following

exposure, it is clear that radiation-induced reversions continue to appear up to the eleventh division but phage-induced reversions all appear at the first division.

Table 6 shows results which appear to me to be difficult to account for, according to the current interpretation of transduction, and to be somewhat more easily understood if we assume a general mutagenic effect of bacteriophage.

TABLE 6  
*One-step reversion isolated after exposure to cell filtrates*

STRAIN NO.	LYSOGENIC	CELL SYMBOL	FILTRATE SYMBOL	REQUIREMENTS	
				(1)	(2)
533-480-57	—	B	b	Leucine and histidine	
549-59-19	+	A2	a2	Proline and threonine	
549-8-13	+	A3	a3	Cysteine and proline	

CELLS	EXPOSED TO FILTRATE	NO. OF REVERSIONS PER PLATE FROM ABOVE REQUIREMENTS TO WILD TYPE	
		(1)	(2)
B	A3	237	34
	a3 b	0	0
	b a3	87	12
A2	b	0	0
	a3	0	0
	a3 b	1	150
	b a3	0	0
A3	a3	0	0
	a3 b	0	0
	b a3	0	0

Cells are exposed by suspension in the filtrate for one hour or more, then centrifuged and resuspended in the culture medium. When a double exposure to filtrate is indicated, it is made by growing the second culture in the initial filtrate overnight and recentrifuging. First, as the table shows, *B* cells (nonlysogenic) show reversions to both leucineless and histidineless following exposure to phage carrying filtrate (*a3*). This reversion-producing effect is prevented if the phage-carrying filtrate is subsequently exposed to the *B* cells and



retested (*a3 b*). This seems difficult to account for on the current transduction scheme.

Second, the phage-bearing filtrate (*a3*) produces reversions of threonineless in the *A2* (lysogenic) cells only if followed by exposure to *B* cells (*a3 b*). This is a typical case of transduction, but it seems strange that it should not act also to produce an increase in threonineless reversions. A difference in the sensitivity to a mutagenic agent of different genes is frequently encountered. The results with *A3* cells, the source of the phage, are perhaps to be expected on either interpretation.

It appears to me that such results, together with the many nonspecific effects already reported in the antigenic studies of Stoker, Zinder, and Lederberg, are most easily interpreted on the view of a general mutagenic effect of products released from cells lysed by phage.

[*July 1954.* After examining table 6 in manuscript, Dr. Zinder suggests that the data do not contradict the transduction pattern, but indeed may confirm it. The results are not inconsistent with the assumption that the phage carries genetic units only from the last strain exposed to it, whether A or B. If subsequent tests conform to this behavior, our results must be interpreted as additional independent cases of transduction rather than mutagenesis.]

LENNOX: Dr. G. Bertani and I have recently demonstrated transduction in some strains of *Escherichia coli*, using sterile lysates of the temperate bacteriophage P1 which was isolated originally from *E. coli* strain Lisbonne. Three different genetic characters have been so far transduced: arginine-requiring to arginine independence; galactose-nonfermenting to galactose-fermenting — both in *E. coli* strain C; and streptomycin dependence to independence in *E. coli* strain B. The lysate of P1 was prepared on a strain of *Shigella dysenteriae*.

DELAMATER: It seems to me to be pertinent to mention that during the last 2 years *Salmonella typhosa* has been studied in considerable detail in our laboratory by Minsavage. The nuclear activity in this organism appears to follow the

pattern that has been previously described as mitotic. As in *Bacillus megatherium*, the chromosomes appear to be continuously confined within a definable membrane and the centrioles appear to reside outside the membrane.

The action of colchicine on the nuclear apparatus of this organism has been studied extensively. In addition to a demonstration of inhibition of mitosis in about 10% of the cells, it has been possible to define a "transient polyploidy" comparable to what has been observed in *B. megatherium* when this organism is exposed to terramycin. If we accept the work of Levan and follow his interpretation of the effect of colchicine as indicating a mitotic process, then the present evidence supports the view that a mitotic mechanism occurs in the bacteria studied.

In *Salmonella*, as in *E. coli*, three discrete chromosomal bodies have been observed. Whether this number constitutes the real haploid chromosome number cannot yet be said. As with the other bacteria studied, the chromosomes in *S. typhosa* likewise undergo an elongation and contraction during the mitotic cycle.

With reference to Dr. Hotchkiss' paper, Dr. Hotchkiss kindly sent me a strain of *Pneumococcus* for cytologic study. Unfortunately, his organism is too small to permit visualization of any intrinsic structure within the nucleus.

LINDEGREN: I should like to ask Dr. Zinder and Dr. Hotchkiss how they reconcile the conflict between classical genetics and the phenomenon of transduction and transformation. It seems quite clear that Tatum and Lederberg's experiments on hybridizing colon bacilli would not have been possible if transduction or transformation occurred in the colon bacilli. The DNA from one parent is in direct contact with the DNA from the other in a hybrid and one might expect positive transduction to occur; all the progeny should be relatively identical.

In the current experiments, exposure of an extract from the chromosome of one parent transforms the other parent.

Do you attempt to reconcile this picture to classical genetics, or what is your reaction?

DEMEREK: Last fall we started to work with *Salmonella*, since in that organism it is possible to determine whether or not two similar mutants are allelic, using transduction tests devised by Zinder and Lederberg. Our main interest is the study of spontaneous and induced mutability in genes, and in this problem information about the allelic relations of similar mutants is of considerable importance.

The determination of allelism is very simple and positive. For example, if we wish to find out if two cystineless strains which are independent in origin are allelic, temperate phage is raised on each of them. If they are not alleles, the temperate phage raised on one strain will transduce the other, and vice versa; whereas if they are allelic, no transduction will take place.

In our experiments with strain LT2 of *S. typhimurium* (in experiments with galactose-fermentation mutants we also used strain LT7) obtained from Zinder, we have collected about 130 independently originating nutritional deficiencies. The collection includes 36 cystineless, 24 prolineless, 24 serineless, 18 histidineless, and 9 tryptophanless mutants. Transduction studies for allelism revealed an interesting series of groupings. For example, we found that *cystineless*-20 (*cys*-20) did not give any transduction with *cys*-1, -3, -5, -13, -21, or -22; thus it is allelic to all of them. Similarly, *cys*-3 and *cys*-5 proved to be allelic to each other. But transduction took place among *cys*-1, -13, -21, and -22, and between each of these and *cys*-3 and *cys*-5. However, the number of transductions observed between members of this group was considerably smaller than the number of observed when we used phage raised on the wild-type strain of bacteria or on any other cystineless strain not belonging to this group. For example, in experiments with similar numbers of bacteria and phage particles, *cys*-1 gave 3 transductions with phage from *cys*-3, but 204 transductions with phage from the wild-type strain and 347 with phage from *cys*-8.

In our material we have found four such groups among the 29 cystineless mutants tested, and two groups among 12 prolineless mutants. Our tests have indicated that in one of the cystineless groups the cystine deficiency can be partially satisfied, in each of the strains, by cystathionine, and that in the strains of another group it can be partially satisfied by either methionine, homocysteine, homoserine, or cystathionine.

These results favor the assumption that the members of each group are allelic to one another, in which case the relatively small number of transductions that were obtained within a group might be due to some process similar to that responsible for recombination between pseudoalleles.

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# GENETIC RECOMBINATION AND REPLICATION IN BACTERIOPHAGE<sup>1</sup>

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## THREE FIGURES

A single bacterium infected with several closely related virus particles may produce a population of progeny viruses some of which receive genetic properties characteristic of different parental particles. This result, first observed by Delbrück and Bailey ('46) and analyzed in terms of genetic recombination by Hershey and Rotman ('48, '49), provides the reason for discussing bacteriophage genetics in a symposium devoted to the understanding of genetic recombination. Whether recombinations in macroorganisms and in phage depend on similar phenomena, or whether indeed they have anything in common, remains to be determined. The purpose of this paper is to give a summary account of the facts of phage recombination and a brief description of a currently acceptable model for this phenomenon. To this review will be added a few recent and preliminary experiments designed to restrict further the present model.

Only a very limited group of lytic phages will be discussed here for the simple reason that it is the only group about

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The remainder of the recent experiments and most of the analyses were completed at the University of Rochester and supported by a research grant, C-2306, from the National Cancer Institute of the National Institutes of Health, Public Health Service.

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which sufficient information is available to restrict models which might be formulated. The so-called T-even group (Benzer *et al.*, '50) of phages active against *Escherichia coli* strain B will be considered, and from this group phages T2 and T4 exclusively will be dealt with. These two phages are sufficiently closely related that, in general, facts obtained with one appear to be applicable to the other. For example, they are serologically related, but distinguishable; they are indistinguishable in the electron microscope; perhaps more crucial to the present discussion, they can grow simultaneously in the same cell (with limited interference, Delbrück and Bailey, '46) and produce genetic recombinants with each other. Other points of similarity or difference will be mentioned where they apply.

Perhaps the only method which need be mentioned is that of making a cross. This term refers to infection of bacteria under such conditions that two genetically different parental particles will be reproduced within the same cell. With appropriate genetic markers, recombinants can be identified by their host range (Luria, '45) and by their plaque morphology (Hershey and Rotman, '49; Doermann and Hill, '53). Further information on the materials and methods may be had by consulting any of several reviews (Benzer *et al.*, '50; Adams, '50).

#### THE GENETIC STRUCTURE AND INFORMATION ABOUT ITS REPLICATION

Experiments have yielded several facts pertaining to the genetic structure and its replication. The first, and perhaps the most fundamental result concerns the recombination values obtained when crosses are made with stocks containing the various genetic markers. Recombination values from both 2- and 3-factor crosses in T2 as well as in T4 can be summarized in terms of genetic maps based on the assumption that the markers have a definite location in a linear structure (Hershey and Rotman, '48, '49; Visconti and Delbrück, '53; Doermann and Hill, '53; Doermann, '53). The slight negative



interference apparent in the data will be discussed subsequently. It is perhaps sufficient to say that in both T2 and T4 three linkage groups have been found, and that by placing *r* (*rapid lysis*) and *m* (*minute*) loci from T2 into T4, it has been possible to obtain evidence that the linkage structures in one phage have homologous counterparts in the other (Doermann and Hill, '53).

One significant experimental fact is available concerning the kinetics of reproduction of these genetic structures. Luria ('51), in examining the distribution of spontaneous mutants among individual host cells, found that the mutants were not distributed randomly among the individual bacteria, but clonally. The sizes of the individual clones fell into an exponential distribution, with many bursts containing a single mutant, fewer bursts containing two, still fewer three, and so on. The data clearly rule out all hypotheses which hold that the daughter genetic structures are produced exclusively on templates associated with the original infecting particles. They leave no doubt that the daughter elements may themselves act as templates.

Investigations of the last few years have made it clear, however, that although replication of the genetic structures involves steps of an exponential nature, the whole infectious virus particle is not being reproduced by serial fissions. Rather, the process of replication is going on at a time when the virus particle is in a noninfectious state, the vegetative state. The existence of this state as distinct from the infectious state can be inferred from the following experiments. A bacterium may be infected with several phage particles and soon thereafter broken up and its virus contents assayed. The bacterium, had it not been disrupted, would have produced a normal crop of phage; but in spite of this no particles can be found within it during the first half of the intracellular period. The infecting particles are evidently incapable of repeating the act of infection (Doermann, '52). When this experiment is performed with bacteria infected with two types of phage differing from each other by two or more genetic

factors, it is further found that the earliest obtainable infectious phage crop already contains genetic recombinants (Doermann, '53). The conclusion cannot easily be avoided that genetic recombination had occurred when the virus particles were in the vegetative state.

The physical explanation of the change of state upon infection is provided by the experiments of Hershey and Chase ('52) who showed that the sulfur-containing protein membrane of the phage remains attached to the outside of the bacterium and may, shortly after infection, be removed from the infected cell without affecting the phage production of the cell. The phosphorus-containing fraction, largely deoxyribonucleic acid (DNA), enters the cell and apparently presides over the construction of new virus.

After the discovery that genetic recombination must occur during the vegetative state, it can be further deduced that multiplication of genetic structures must also occur during that stage. Hershey and Rotman ('49) showed that recombinants are distributed randomly among individual bacteria and not clonally. The fact, noted previously, that the first infectious particles formed in cells may already be recombinants, implies that these early recombinants must grow into clones on any hypothesis which permits exponential replication of infectious particles.

Since all practically usable methods of enumerating bacteriophage particles and of differentiating between genetic types depend on the infectious property of the virus, the vegetative state masks both the phenomenon of replication and the phenomenon of recombination. It is precisely these processes which one would like to study in phage. It therefore becomes necessary to turn to less direct experiments which depend on extrapolating from the infectious population within a bacterium to the vegetative population.

This approach has given several results which will assist in formulating a model for genetic recombination in phage. One is the observation that a drift in the proportion of intracellular recombinants occurs with time. When the intra-

cellular population is sampled at intervals by premature, normal, or delayed lysis, it is found that the recombinant fraction increases, approaching genetic equilibrium. For example, the percentage of recombinants for  $r_{13}$  and  $h$  in T2H is, according to Levinthal and Visconti ('53), about 2.6 at normal lysis, but rises to 11.8% when lysis is inhibited. Similarly, in the cross  $tu_{42} \times tu_{44}$  in T4 the proportion of recombinants at normal lysis is about 11.5%, but if the yield is obtained by early premature lysis only 6% recombinants are found (Doermann, '53).

Another series of experiments leads to the conclusion that the acts by which recombination is accomplished (hereafter called matings) occur repetitively. It was already noted in the first recombination study by Hershey and Rotman ('48) that when a cell is infected with three types of phage,  $a++$ ,  $+b+$ , and  $++c$ , the yield includes recombinants of the  $abc$  genotype, containing markers from each of three parents. That triparental recombinants of the  $abc$  genotype are not a rare occurrence was subsequently made clear by Hershey and Chase ('51), who showed that the proportion of such recombinants approaches genetic equilibrium.

Another result indicative of repetitive mating comes from two-factor crosses in which very unequal multiplicities of the parental types are used. Under these conditions, each of the recombinant classes was found in greater proportion than the minority parent (Doermann, '53). Clearly, this result could not come about from a single biparental mating.

#### THE VISCONTI-DELBRÜCK THEORY

On the basis of the foregoing information and additional extrapolative experiments, Visconti and Delbrück ('53) formulated an algebraic theory to account for genetic recombination in phage. They visualized the life cycle of the virus as follows: Upon infecting the host cell, the parental phages are transformed from the infectious state to the vegetative state. In this condition they multiply exponentially until they form a moderate-sized pool of vegetative particles. As the

pool gets larger, the individual vegetative phage particles mate with one another and form genetic recombinants. The processes of replication, mating, and recombination continue until the cellular structure of the host is dispersed at lysis. Soon after mating begins in the pool, particles are removed from it and transformed into infectious phages, staying dormant and accumulating in that condition until they are liberated by lysis of the cell.

The theory has three parameters: the number of repeated mating encountered per progeny particle; the probability of recombination with respect to any pair of genetic markers in any one round of mating; and the relative multiplicity of infection of the parental types. Visconti and Delbrück measured the average number of matings per particle for T2 in a cross involving three unlinked factors with the multiplicity of the two parents very unequal. Measuring the disappearance of the minority parent gave an estimate of about five matings per particle in a normal T2 latent period and indicated that the five matings were spread randomly in time and did not occur synchronously among the various particles. From genetic experiments Levinthal and Visconti ('53) estimated that the pool in which matings occur consists of about thirty vegetative particles.

In addition to unifying and explaining satisfactorily the previously described heterogeneous information on the processes of replication and recombination, the theory predicts another well-established result, the phenomenon of negative interference. This prediction can easily be visualized qualitatively if one imagines that the progeny is made up of two equal classes, one in which no matings at all have occurred and the other in which the individual particles have mated once. In the latter class the double recombinants may be precisely as expected on the basis of the frequencies of recombination in regions one and two. When the former class is added to the data, however, the observed individual recombination values will be reduced by a factor of 2. The expected number of doubles will be reduced by a factor of 4, while the observed



number of doubles will be reduced by a factor of only 2. Consequently, the latter will be too high by a factor of 2, showing apparent negative interference. A qualitatively similar result will be observed if the matings are randomly distributed around any average value.

Experimentally, negative interference has invariably been observed in both 2-factor (Doermann and Hill, '53) and in 3-factor crosses (Hershey and Rotman, '48; Visconti and Delbrück, '53; Doermann, '53). That the theory quantitatively accounts for these observations is indicated by the complete removal of the negative interference apparent in 2-factor mapping experiments when the value  $p$ , the probability of recombination per mating, is mapped. This value, calculated on the basis of Visconti-Delbrück kinetics, is freed from the disturbances caused by the fact that the population, sampled at any time, is a heterogeneous mixture of particles with different amounts of mating experience.

#### THE ROLE OF THE HETEROZYGOTE

In general, the Visconti-Delbrück theory appears to describe well the kinetic aspects of recombination in phage. Since it was formulated, however, at least one major contribution has specified it further. One of the shortcomings of the original theory is that it contains no feature enabling it to account for the so-called heterozygotes of phage discovered by Hershey and Chase ('51). These structures, in genetic terms, appear to be diploid for small segments of a linkage group. The probability that a particle heterozygous for one locus will also be heterozygous for a second depends directly on the closeness of the linkage of the two. For any given marker, the probability of finding it heterozygous in a progeny phage particle from the appropriate cross is about 2% in T2H, and this value is constant throughout the period when infectious phage may be found within the cell. Furthermore, when the heterozygote is permitted to infect a bacterial cell, the progeny formed in this bacterium show no unusually high proportion of heterozygotes, but again yield only 2%.

These results suggested to Levinthal ('54) that the structure of the heterozygote might be visualized in two ways: first, it might consist of a small piece of haploid genetic material attached to the side of the normal linkage structure; or second, it might be two fractional linkage groups together making up the whole, but attached to each other by an overlapping region. Levinthal performed an ingenious experiment to distinguish the two possibilities. After making a cross involving three linked factors, he analyzed heterozygotes for the centrally located marker. Clearly, if a separate piece of genetic material were attached to the normal structure, the latter should, in the majority of cases, be parental with respect to the terminal markers. If two partial structures are connected by an overlap, the genotype should usually be recombinant with respect to the terminal markers. The experiments indicated unambiguously that the first model may be ruled out, but that the second is a satisfactory one to explain the structure of the phage heterozygote.

From these results it also became clear that the heterozygous particle, when reproduced, yielded recombinants with respect to the three markers concerned. Levinthal now attacked the question whether the heterozygotes might be the source of all recombinants within linkage groups. The following assumptions, based on the previously described information, were made: (1) that the Visconti-Delbrück theory is correct in principle; (2) that the production of heterozygotes involves a mating of two unlike phage particles, and that these produce a new structure containing a short overlap; (3) that heterozygotes do not reproduce themselves, but yield nonheterozygous progeny at the same rate as normal vegetative particles; and (4) that the overlap is equally likely to occur at any point in the genetic structure. With these assumptions Levinthal was able to show that heterozygotes can account for all recombinants involving linked markers.

The principle modification which this brings about in the Visconti-Delbrück theory is that, by the formation of the heterozygote, a new structure is interposed between the

parents and the recombinant. The new structure is thought of as forming the recombinants. Thus the formation of the recombinant is thought to imply the cooperative formation of a new particle, rather than a simple exchange during a mating between genetically complete vegetative phages.

#### NEW EXPERIMENTS WITH IRRADIATED PHAGE

With the hope of adding detail to the picture of replication and recombination, some recent experiments with irradiated phage will be reported. Since, with phage, it is not possible to use many genetic markers simultaneously, owing to the scarcity of distinguishable phenotypes and to epistatic effects, it was hoped that at least some of the lethal radiation damages could be shown to be localized in the genetic structure of the phage particle. If so, they should constitute a powerful tool in studying phage genetics.

Extensive experiments along this line have already been described. Luria ('47) and Luria and Dulbecco ('49) proposed genetic recombination as a mechanism by which non-homologous lethal damages from ultraviolet radiation are eliminated when two or more inactivated phages cooperatively form a progeny of active phage (multiplicity reactivation). On the basis of subsequent experiments by Dulbecco ('52), the hypothesis was abandoned (Luria, '52). Although the present experiments are not entirely new in principle, they incorporate certain technical differences which make additional conclusions possible. These suggest that the hypothesis of elimination of lethals by genetic recombination may have been abandoned prematurely.

The following experiment may be used to test whether radiation damages are localized in the genetic structure. Extracellular phage of the wild genotype is irradiated. The inactivated particles are adsorbed to bacteria under conditions in which 20% of the bacteria receive one phage. Simultaneously, unirradiated particles carrying three mutant genetic markers are adsorbed to the same bacteria with a multiplicity of 2-3. The adsorption is carried on in the

presence of cyanide so that, on subsequent dilution into cyanide-free medium, all the phage particles resume their intracellular course at the same time. Single bacteria having only one irradiated particle and several "carrier" particles are isolated by dilution before burst so that their progenies may be analyzed separately. These may then be scored for the presence or absence of the genetically marked loci of the irradiated parent, and the frequencies of the individual markers in each burst may be noted.

Two types of experiment have been done with this procedure: (1) a more or less thorough series involving three unlinked genetic loci, with analysis of bursts following various doses of radiation; and (2) several preliminary tests with linked loci. In both cases wild-type phage was the irradiated parent. From these experiments, several facts emerge: (1) A given marker originally present in the irradiated parent may be absent from a burst even though the other markers may be present in appreciable frequency (table 1). The frequency of marker absences (which will be called "knock-outs") is related directly to the dose of ultraviolet radiation. (2) Simultaneous inactivation of two linked markers occurs more frequently than is accountable by chance. Such double inactivations bear a direct relation to the closeness of the linkage (table 2). (3) Unlinked markers, on the other hand, appear to be inactivated independently of one another. This will be taken up in more detail subsequently. (4) When one of a pair of linked markers is absent from a burst, the marker linked to it shows, on the average, a lower frequency in the burst than does an independent marker (table 3).

The hypothesis which is proposed to account for the results described is that part, at least, of the ultraviolet damages are located in the genetic structure, and that genetic recombination may rescue a marker from the damaged unit. The last three experimental facts given in the preceding paragraph appear to justify tentative acceptance of the general hypothesis. Further analysis of the experiments involving the unlinked markers may add further details. The analysis will



proceed along two lines. First, the frequency of knockouts as a function of dose will be examined more closely. Second, the yield of surviving markers in the individual bursts will be studied.

TABLE 1

*Yield of wild-type loci in single bursts following a 2-minute dose  
(4 hits) of ultraviolet radiation*

Wild-type phage was irradiated for 2 minutes and adsorbed to bacteria (multiplicity ca. 0.3) simultaneously with carrier phage of the genotype  $m_{42}r_{42}tu_{42b}$ . Single bursts were scored. Of 125 mixed bursts, 41 lacked a single wild-type marker and 9 lacked 2. Twenty representative bursts are given, 10 with single knockouts and 10 with no knockouts.

Burst no.	FREQUENCY OF GENOTYPES							Absent
	+++	$m+r+$	$++tu$	$m++$	$+rtu$	$m+tu$	$+r+$	
1-3	0	26	0	1	0	1	0	$m^+$
1-6	0	0	0	0	2	21	0	$tu^+$
1-11	0	3	0	1	0	51	0	$m^+$
1-13	0	0	0	0	8	48	0	$tu^+$
1-14	0	0	13	0	11	61	0	$tu^+$
1-17	0	0	35	0	59	32	0	$tu^+$
2-3	0	4	0	0	0	5	0	$tu^+$
2-5	0	13	0	0	29	0	36	$r^+$
2-6	0	1	0	0	61	0	3	$r^+$
2-9	0	0	7	0	6	6	0	$tu^+$
1-1	0	21	0	1	11	0	4	..
1-2	0	1	0	0	6	1	0	..
1-5	1	27	0	0	0	0	0	..
1-7	0	5	3	0	7	0	8	..
1-9	0	6	3	1	14	14	0	..
1-12	2	13	4	0	42	19	1	..
1-15	0	0	27	0	25	49	1	..
1-16	8	42	8	10	52	29	32	..
1-18	0	13	0	2	13	3	4	..
1-19	0	16	1	0	19	2	7	..

*Analysis of frequency of knockouts.* In estimating the total frequency of knockouts, it is immediately obvious that a particle in which all three markers have been inactivated escapes recognition. If two assumptions are made at the outset, the survival probability of a genetic locus can nevertheless

be calculated. The assumptions are: (1) that the individual markers are inactivated independently of one another, and (2) that the three markers show equal sensitivity to inactivation by ultraviolet radiation. The individual bursts may be separated into four classes, one in which all three loci survive,

TABLE 2

*Inactivation of linked loci by a single low dose of ultraviolet radiation*

Wild-type phage was irradiated with ultraviolet light to a survival of 0.005 (5.3 hits). It was adsorbed to bacteria at a low multiplicity. Simultaneously, phage carrying three linked markers  $m_{42}r_{51}tu_{41}$  was adsorbed. The loci are linked in the order  $m, r, tu$ , with about 12 recombination units between  $m$  and  $r$  and about 20 between  $r$  and  $tu$  (Doermann, '53). If the loci are inactivated independently of one another, and  $p$  is the probability of survival of any locus, then the bursts with no knockouts should be equal to  $p^3$ , the bursts with  $m^+$ ,  $r^+$ , or  $tu^+$  absent should equal to  $p^2(1-p)$ , and the bursts with any given pair of markers absent should be equal to  $p(1-p)^2$ . The value of  $(1-p)$  is the sum of all the knockouts divided by the total loci and is equal to 49/318, which equals 0.154. The triple knockout would be negligible on the hypothesis of independence.

FREQUENCY OF BURSTS		
Locus absent	Expected <sup>a</sup>	Found <sup>a</sup>
None	64	69
$m^+$	12	2
$r^+$	12	8
$tu^+$	12	15
$m^+$ and $r^+$	2	9
$m^+$ and $tu^+$	2	1
$r^+$ and $tu^+$	2	2
Total	106	106

<sup>a</sup> When the  $\chi^2$  test is used to compare the observed with the expected values, a deviation is found which is significant beyond the 1% level. The deviation arises primarily from the fact that the closely linked  $m^+$  and  $r^+$  loci are inactivated together in excess of the expected value, and that the frequency with which they are separately inactivated is lower than expected.

one in which any pair survives, one with a single surviving locus, and one in which all three loci have been inactivated. If  $p$  is the probability of survival of a given marker, then the expression

$$p^3 + 3p^2(1-p) + 3p(1-p)^2 + (1-p)^3 = 1 \quad (1)$$

includes all classes in the order given above,  $p^3$  representing

the class with all three markers surviving,  $3p^2(1 - p)$  the class with only two surviving, etc. The best estimate of  $p$  for each dose of radiation can be obtained by application of the maximum likelihood function to the first three classes. The various estimates of  $p$  may then be plotted, and the survival curve of a genetic locus, as a function of dose of ultraviolet radiation, may then be drawn (fig. 1). It is seen that the probability of survival decreases logarithmically with dose. A similar result was observed by Luria ('52).

TABLE 3

*Influence of a knockout on the burst size of a linked surviving locus*

Wild-type phage was irradiated with low dose (6 hits) and adsorbed to bacteria at low multiplicity. Simultaneously, carrier phage marked with  $m_{43}r_{51}tu_{42b}$  was adsorbed. Two loci  $m_{43}$  and  $r_{51}$  are linked to each other and are independent of  $tu_{42b}$  (Doermann, '53). The bursts with either linked marker inactivated, but with the other two loci surviving, were selected for analysis. The total yield of the linked survivors was compared with the total yield of  $tu^+$  in the selected bursts. For a control, bursts with no knockouts were selected, and the total yields of  $m^+$  and  $r^+$  were averaged and compared to the total yields of  $tu^+$ .

	TOTAL YIELDS			
	Experiment with knockouts		Control without knockouts	
	$m^+$ or $r^+$	$tu^+$	$(m^+ + r^+)/2$	$tu^+$
Experiment 1	173	392	660	742
Experiment 2	543	748	2148	1967
Total	716	1140	2808	2709
Ratio of linked to unlinked loci	0.63		1.04	

On the assumption that the three loci have equal sensitivity, the ten experiments may be examined individually to see whether they fit the classes predicted by the hypothesis of independent inactivation of unlinked markers. That is, they may be tested to see whether the classes with no knockout, with one knockout, and with two knockouts fit the predicted distribution given by (1). Although three of the experiments show considerable deviation, the other seven fit exceptionally well, and since no trend is apparent in the three discordant

experiments, the hypothesis of independence may be tentatively accepted until more extensive data prove otherwise.

It should be pointed out that the assumption of equal sensitivity of the markers is only one of convenience and could be eliminated by calculating individual values of  $p$  for

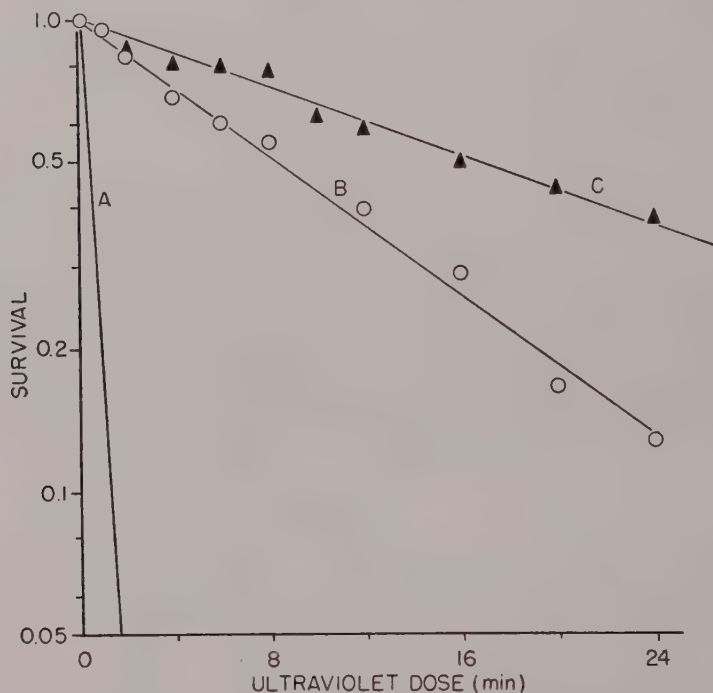


Fig. 1 Survival of plaque formation, genetic locus, and bacteria-killing ability of phage as a function of dose of ultraviolet.

Curve A gives the plaque-forming ability and presumably represents the total radiation damages. Curve B shows the survival of a genetic locus (see text). Curve C follows the loss of bacteria-killing ability. The dose is approximately two lethal hits per minute.

the three loci. The data are not sufficiently large to justify such calculations. It is worthy of note that there is some indication in the data that, of the three markers used,  $tu_{42+}$  is slightly less sensitive to ultraviolet radiation than either  $r_{48+}$  or  $m_{42+}$ .



The survival curve of the locus may be used to obtain a minimum estimate of the proportion of ultraviolet damage which is located in the genetic structure. Comparison of the slope of the survival curve for plaque-forming ability (which measures the total lethal damage) with the slope of the curve for marker survival (fig. 1) shows that 4% of the total ultraviolet damages inactivate a given locus. Since three loci which sustain damages independently have been averaged to give this measurement, at least 12% of the total damages have been localized in the genetic structure. If the data from linked markers are examined (table 2), it is apparent that two markers separated by 12–20 recombination units are inactivated more or less independently of one another. When the total recombination map of T4 (Doermann, '53) is taken into account, at least six independently inactivated locations can be assigned: one in linkage group I  $r_{48+}$  is one of the loci used in the present experiments); two in linkage group II (36 recombination units mapped); and three in linkage group III (56 recombination units mapped). Each of the six locations should have a cross section of 4% of the total phage. Thus 24% of the total hits appears to be a safe minimum estimate of the proportion of ultraviolet damages which are absorbed by the genetic structure. The value will rise, of course, if more extensive mapping experiments add new areas to the currently known linkage groups. It may rise or fall with a more reliable estimate of the recombination distance required for linked markers to show independence with respect to ultraviolet inactivation.

In considering the minimum estimate to be 24% of the total damage, the following point also should be brought to attention: the estimate of genetic damage is not influenced by particles which are for some other reason incapable of making a genetic contribution. For example, 2% of the total ultraviolet damages prevent the phage from killing bacteria (fig. 1) and presumably from contributing genetic material. The estimate of 24% depends only on the proportion of particles

contributing one, two, or three genetic loci and thus would be independent of the nonkilling particles.

*Analysis of burst size of irradiated loci.* To gain a general impression of the influence of ultraviolet radiation on burst size, the yield of a given marker per bacterium infected with an irradiated phage particle may be calculated (fig. 2, curve

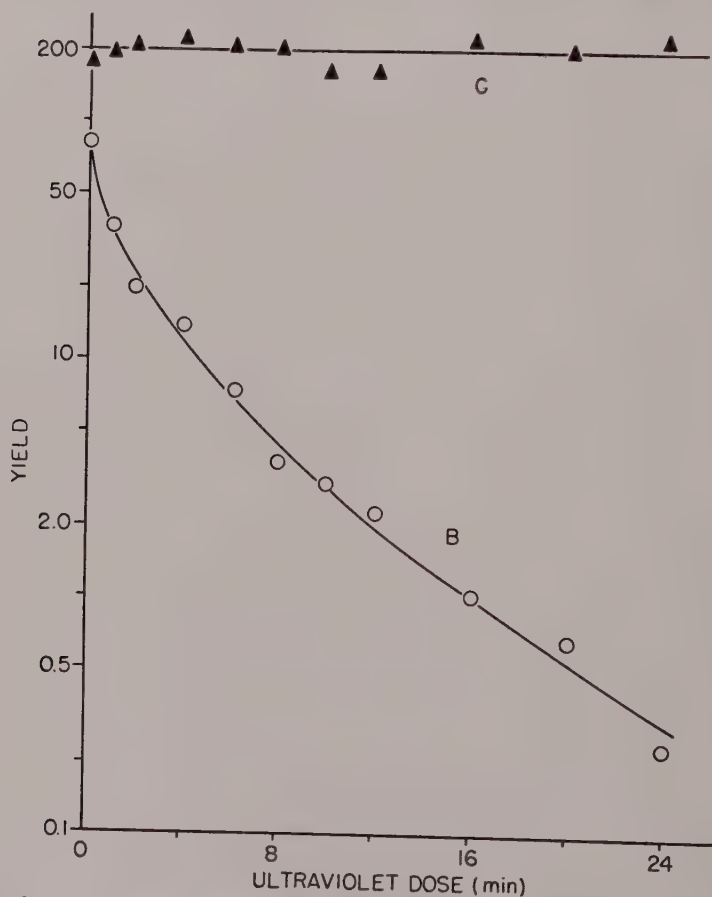


Fig. 2 Burst size as a function of dose of ultraviolet radiation. The average yield of infectious particles showing a particular irradiated locus is shown in curve B. The number of bacteria lacking all 3 irradiated loci is calculated from the survival curve of locus (see figure 1), assuming the loci are independently inactivated. Curve C gives the average combined yield per bacterium of all types of phage: carrier, irradiated parent, and recombinants.

B). Although the total phage yield per bacterium is not reduced (fig. 2, curve C), a marked depression in the average burst size of the irradiated loci is noted with increasing dose, much greater than is accounted for by the total knockouts. Further examination of the burst size distribution of irradiated loci reveals a second fact. To illustrate it, the accumulated distribution may be plotted as was done by Luria ('51, fig. 1) in studying the burst size frequency distribution of spontaneous mutants. In the present data, the logarithm of the burst size of the genetic markers ( $x$ ) is plotted against the logarithm of the number of bursts containing  $x$  or more individuals of a rescued locus ( $Y_x$ ). Figure 3 shows the accumulated distribution curves for surviving loci in the experiments where the wild-type phage were exposed to ultraviolet for 20, 16, 12, and 0 minutes. Clearly, the distribution with high doses is strikingly similar to the distribution found by Luria for spontaneous mutants.

Both of the results described are compatible with the hypothesis that ultraviolet damage is located in the genetic structure and that genetic recombination rescues loci from these damages. The first observation, general depression with increasing dose, would result from the increasing number of hits which necessitate a more and more precisely located recombination for effecting rescue. Both late rescues and lower frequency of rescues per intrabacterial cycle would reduce the burst size of the rescue locus.

The meaning of the second observation, that of the Luria distribution at high dose, is not clear at this time. The result indicates that there must be an increase in the probability of rescue with intracellular time, so that half of the rescues occur in the terminal generation, half of the remainder in the next-to-last generation, etc. The requirement might be satisfied by exponential increase of the carrier population, thereby increasing the chance of a successful recombination. It might, on the other hand, be satisfied by exponential multiplication of the irradiated structure. At present, no clear-cut decision can be made between the two alternatives.

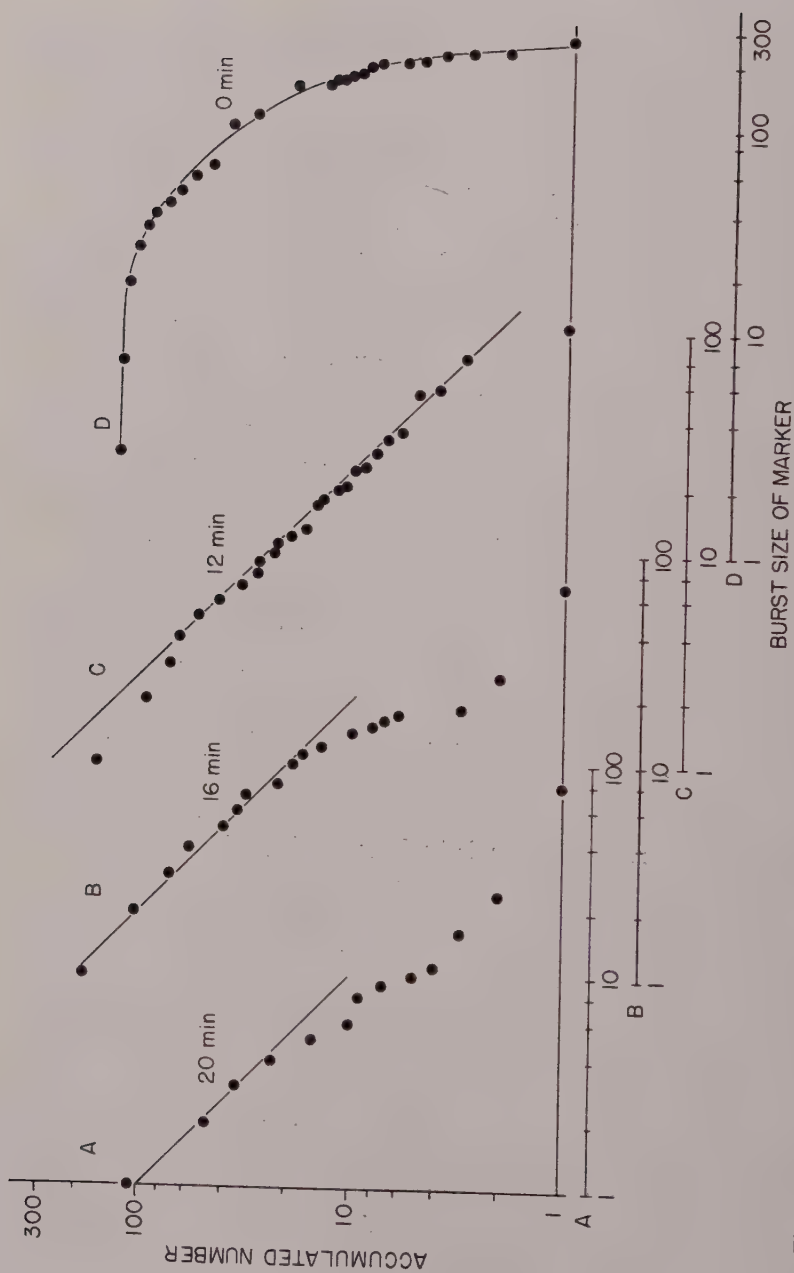


Fig. 3 The distribution of yields of rescued loci among single bursts. The numbers next to the curves indicate the dose of radiation in minutes. The solid lines have slopes of  $-1$ .



Two facts which bear on this question should, however, be mentioned. First, genetic evidence presented by Levinthal and Visconti ('53) is compatible with the notion that the vegetative pool remains at a more or less constant level of thirty particles during the majority of the period of replication and recombination. The compatibility does not, however, constitute sufficiently decisive evidence to rule out the possibility of an exponentially increasing carrier population. Second, chemical evidence (Hershey, Hudis, and Chase, '53) indicates that the phage DNA of the vegetative pool remains more or less constant between 50 and 100 phage equivalents during the second half of the latent period. If DNA is the genetic material (or an essential part of it), and if the whole of the DNA of one phage particle consists of essential genetic structure, the alternative involving growth of the carrier population could be ruled out. Although neither the genetic nor the chemical evidence is compelling, the two together suggest investigating whether a consistent hypothesis could be developed on the assumption of exponential growth of the genome of the irradiated phage.

*Replication of radiation-inactivated material.* It now seems clear that an appreciable fraction of the total ultraviolet hits is located in the linkage structure and that genetic recombination is responsible for rescuing the undamaged material. It follows from the high efficiency of multiplicity reactivation (MR) (Luria, '47) that genetic material of the irradiated particle is replicated. The alternative that two multihit particles can undergo all the necessary recombinations to make a complete reactivated particle seems less probable.

Luria ('47) and Luria and Dulbecco (49) have pointed out that the high efficiency of MR suggests that lethals themselves are not incorporated into newly formed phage particles. To test whether a population of phage produced by MR contains particles carrying lethals, a sample of the wild-type phage irradiated for 24 minutes (fifty hits) was mixed with bacteria so that, on the average, several particles infected each bacterium. The resultant stock was subjected to two tests. One

was to adsorb it to bacteria along with carrier phage and examine single bursts for knockouts. None were found. The other was to compare the plaque-forming titer and the bacteria-killing titer. The two were found to be equal. Hence, the MR stock contained no infectious particles which were not genetically complete, indicating, in agreement with Luria ('47) and with Luria and Dulbecco ('49), that if the inactivated material is reproduced at all, it is perfectly excluded from the infectious progeny.

Additional information which indicates that the damaged material is not replicated comes from examination of the average burst size. On the assumption that lethals are replicated and mixed into the vegetative population, it could be predicted that, in view of the high frequency of recombination between the unlinked genetic loci, heavily irradiated phages would contribute lethals to nearly all vegetative particles. The yield from such bacteria should be greatly reduced. Curve C in figure 2 shows that the burst size was not reduced even when the irradiated parent had received fifty lethal hits (24 minutes of ultraviolet). In that experiment, the average multiplicity of the ultraviolet parent was three, one of which had retained its killing ability. Therefore, 63% ( $1-e^{-1}$ ) of the host cells received one or more phages which were capable of killing and which carried a minimum of 12.5 lethal hits in their genetic structure. If the lethals had been replicated, they should have reduced the burst size of 63% of the cells to nearly zero. Since no evidence was found for reduced burst size, it is concluded that lethals are not replicated.

#### THE PARTIAL-REPLICA HYPOTHESIS

Analysis of the experiments involving unlinked loci has added certain tentative pieces of information to the hypothesis of rescue by recombination and its connection with the problem of replication. The discovery that, following high doses of radiation, rescued loci are distributed clonally, to-

gether with the highly efficient occurrence of MR, suggests that part of the irradiated particle multiplies. Three findings, however, indicate that the lethals themselves are not replicated: (1) high efficiency of MR, (2) failure to find lethals in the progeny from MR, and (3) failure to find the average burst size depressed by heavily irradiated infecting particles. The possibility which remains open is that the undamaged part of the irradiated phage multiplies but the damaged part does not. Partial replicas of the linkage groups would then be formed, enter the vegetative pool, and attach themselves to complementary genetic structures before they could be encased in protein membranes and become incorporated into infectious phage.

Supposedly, particles which have not been irradiated multiply similarly. The apparent failure of ultraviolet lethals to be replicated suggests a difference in the replication processes of normal and irradiated phages. In the latter, duplication seems never to extend across the damaged point, with the result that many short replicas are produced, particularly at high doses. Considerably longer replicas would be required in the normal process in order to account for the observed linkage relations.

To say what the partial-replica hypothesis would predict concerning MR would be premature and must await additional experiments and development of a formal theory. It is possible that, in the experiments described, a considerable sector of the total ultraviolet damage is nongenetic and may be circumvented by the fully active carrier particles involved.

In summary, only one point is worthy of mention. Visconti and Delbrück ('53) proposed that recombinants result from matings between genetically complete vegetative phages; the partial-replica hypothesis suggests, rather, that recombinants originate from the union of complementary fractions of the genome. An unambiguous experimental decision between the two alternatives would assist our understanding of replication and recombination in phage more, perhaps, than any

other contribution. Certainly, such a decision is fundamental to the formulation of the ultimate model.

#### ACKNOWLEDGEMENT

The authors are indebted to Dr. A. W. Kimball, Mathematics Panel, Oak Ridge National Laboratory, for assistance with the statistical aspects of this investigation.

#### DISCUSSION

*Chairman HERSHEY:* I should like to express my appreciation of Dr. Doermann's fine paper by giving two reasons for it. Luria's work in 1947 seemed to have shown at last that radiations really produce primary localized damages in genetic material. The same can be said now for Doermann's. Secondly, if radiochemical damage can put genetic material out of commission bit by bit, one ought to be able to measure this damage in chemical as well as genetic terms. Mr. Stahl has one idea how to do this, and we can hope for others.

*STAHL:* Experiments similar in design to those just described by Dr. Doermann for ultraviolet-inactivated phage are being applied to phage inactivated as a result of the disintegration of incorporated  $P^{32}$ . Stent ('53) has shown that the  $P^{32}$  inactivation of phage need not prevent the inactivated phage from contributing genetic markers to the progeny of a mixed infection. In an experiment in which two unlinked loci were followed, he showed that an inactivated phage may contribute both, only one, or neither of its markers, and that the losses of the markers are more or less independent events.

We have been studying more extensively the effects of the disintegration of incorporated  $P^{32}$  on the structure of the phage as described genetically. The likelihood that a disintegration prevents both of two markers from appearing in the progeny formed in a single bacterium has been found to bear an inverse relation to linkage distance. It is hoped that these experiments will permit an estimate of genetic distances in molecular dimensions.



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# RECOMBINATION MECHANISMS IN BACTERIA

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ONE FIGURE

Several mechanisms of genetic recombination have been described or inferred in bacteria. Genetic recombination is taken to include any biological mechanism for the reassortment within one cell lineage of determinants from distinct sources. The present classification (table 1) is based on the scope of the unit of exchange. Most of its categories are exemplified among the bacteria. Cytoplasmic exchange, however, is not yet documented but may be suspected as a corollary of possible "disinfections" (Van Lanen *et al.*, '52; McIlroy *et al.*, '48; Bunting *et al.*, '51).

## GENERAL FEATURES OF RECOMBINATION MECHANISMS

### *Heterokaryosis*

Heterokaryosis is best known among the filamentous fungi for it consists of the coexistence of genetically different nuclei within a single cell or cytoplasmic field (Pontecorvo, '46). The persistent integrity of the constituent nuclei distinguishes heterokaryosis from sex; indeed, genetic exchange (karyogamy) may sometimes intervene without the overt paraphernalia of the sexual stage (Pontecorvo, '53; Papazian, '54). Heterokaryosis may be initiated by mutation within a coenocyte, by deferred nuclear separation after meiosis, or by anastomosis of cells, hyphae, or spores. The first two

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TABLE 1  
*Mechanisms of genetic recombination*

DESIGNATION	UNIT TRANSFERRED	AGENCY	FATE	EXAMPLES IN BACTERIA OR OTHER MICROBES
(1) Sex (karyogamy)	Intact nucleus	Union of cells, hyphae, or more or less spe- cialized gametes or gametangia	Hybrid zygote nucleus may be followed by chromosome segregation, elimination, or crossing over.	Lederberg and Tatum, '53
(2) Heterokaryosis	Intact nucleus	(Same as above)	Intact nuclei in common cytoplasmic field. May be terminated by (1) or by nuclear segregation.	<i>Streptomyces?</i>  (See Pontecorvo, '46)
(3) ?	Intact chromosome	?	?	Hypothetical (See Muller and Pontecorvo, '40)
(4) Genetic transduction	"Chromosome" frag- ment	Chemical preparations of DNA  Bacteriophage	Integration into genotype	Austrian, '52; Zinder and Lederberg, '52
(5) Lysogenic conversion	Phage nucleus (prophage) [May be special case of (4)]	Infective phage	Incorporation unless doomed by bacteriolysis (Bacteriolysis may occur sporadically in later generations.)	Lwoff, '53
(6) Cytoplasmic transfer	Plasmids (viruses, symbionts, plasma- genes, or other ex- tranuclear hereditary factors)	Plasmogamy [usually associated with (1) or (2)]  Intercellular infection	Hereditary symbiosis	Hypothetical for bacteria (See Ephrussi, '53; J. Lederberg, '52; Sonneborn, '50)



modes of heterokaryosis are only incidental to recombination, but cannot be ignored as transient phenomena in customarily multinucleate bacteria (Lederberg, '49a; Witkin, '51). Persistent heterokaryons could be expected only in filiform bacteria such as the actinomycetes.

Sex may be taken as equivalent to karyogamy, the formation of a hybrid zygote from the fusion of two intact "gamete" nuclei. Peripheral to this essential, sexual processes are subject to a variety of classifications based on discordant criteria, giving such distinctions as: isogamy versus heterogamy; monoecy versus dioecy; prezygotic versus postzygotic meiosis; syngamy via conjugation versus copulation versus persistent dikaryophase; germinal versus somatic reduction; meiotic versus mitotic crossing over; autogamy versus exogamy; and many others. The initial act of karyogamy (hybridity) must also be distinguished from chromosome segregation or elimination and from crossing over within chromosome pairs as aspects of sexual recombination cycles.

Among bacteria, the genetic analysis of sex has been carried furthest with *Escherichia coli*, some 5% of the strains tested so far being fertile (Lederberg and Tatum, '53). In addition, similar methods have been applied by various authors to support their tentative claims of sexual recombination in *Achromobacter fischeri* (McElroy and Friedman, '51), *Serratia marcescens* (Belser and Bunting, '54), and *Bacillus megatherium* (Delamater, '53), but not in *Proteus* L forms (Hutchinson and Medill, '54), *Azotobacter agile* (Ziebur and Eisenstark, '51), *Pseudomonas fluorescens* (Lederberg, unpublished), or *Salmonella* (Lederberg, '47b; Zinder and Lederberg, '52). Experiments with *Streptomyces griseus* have been indecisive owing to confusion from heterokaryotic interactions (Lederberg, unpublished), but a tentative suggestion from morphological studies (Klieneberger-Nobel, '47) on the sexual origin of the entire aerial mycelium has no genetic support. Other claims of bacterial sexuality based on suggestive photographs deserve closer genetic attention than has so far been recorded.

*Genetic transduction*

Although the preceding mechanisms are important in relating the fundamental genetic structure of microbes to higher forms, their exposition does not give much leverage on the mechanism of crossing over, which is the fulcrum of this symposium. However, recombination in a number of bacterial species has been found to occur by genetic transduction, a new mechanism which differs from sex by the fragmentary nature of the unit of exchange. That is, transduction is defined as the transmission of a (nuclear) genetic fragment from a donor cell (which in every case so far is destroyed in the process) to a recipient cell which remains intact.

Like sex, transduction may be classified by several criteria. Rudimentary knowledge already distinguishes at least two categories, depending on the agency of transfer: deoxyribonucleic acid (DNA) (*pneumococcus*, Avery *et al.*, '44; *Hemophilus influenzae*, Alexander and Leidy, '51) or carriage by a virus particle (*Salmonella*, Zinder and Lederberg, '52; *E. coli*, Morse, '54). Other categories might depend on the frequency of transmittal, the specific characters that may be or have been transmitted, the persistence of the intermediate heterogenic state, the complexity of the fragments, or the bacterial species. There is little or no indication of transduction in higher forms, but too few experiments have been reported (see Marshak and Walker, '45; Mazia, '49; Klein, '52) to be conclusive in the face of the obvious technical obstacles. Possible further examples of transduction that have not been so fully analyzed are reviewed elsewhere (Lederberg, '48, '49a; Austrian, '52).

Probably inadvertently, a previous discussion in this symposium may have intimated that transduction in the pneumococcus (type transformation) became relevant to recombination only after two or more markers were explicitly followed, and their reassortment noted. But Griffith's experiment ('28) already posed a serious genetic question: How does the unit recombine with the whole? During the following two decades,

the chemical analysis of pneumococcus transformation took precedence over genetic consideration. Some tentative suggestions were adopted that failed to encourage multiple-marker experiments (or were based on their absence) and were therefore barren — “directed mutation” (Dobzhansky, '41) or infection by a presumably cytoplasmic “virus” (Lederberg, '49a, among others). The concept of transduction to explain the pneumococcus transformation and succeeding examples was, however, well stated by Muller ('47): “still viable bacterial chromosomes, or parts of chromosomes . . . might . . . have penetrated the capsuleless bacteria and in part at least have taken root there, perhaps after having undergone a kind of crossing-over with the chromosomes of the host.” Most of the genetic analysis that succeeded this prescription has been based on this point of view, and its success has amply justified the concept.

In the pneumococcus, transduction is mediated by raw DNA, extracted from bacteria that are fragmented with bile salts, and refined by the chemist (McCarty, '46; Austrian, '52). Much insight into the chemical constitution of the genetic reagent has been achieved, and a plausible case has been presented for the sufficiency of deoxyribonucleate alone (as it has for the genetic content of phage). For so crucial a question, however, the standards of proof should be more than ordinarily rigorous (Hershey, '53), and some obstinate doubts on the possible accessory role of protein components will be dissolved only when the non-DNA residues of the preparations are shown to be stoichiometrically disqualified. This standard is admittedly as high as or higher than any in biochemistry but is commensurate with the stature of the conclusions. We have to keep in mind the difficulties in the physicochemical characterization of linear polyelectrolytes which vitiate such criteria as electrophoretic or sedimentational homogeneity, as well as estimates of particle size.

In *Salmonella*, on the other hand, the genetic fragment is embedded in a phage particle, from which it has not been extricated in active form, possibly only because we have not

learned to imitate the faculty of penetration by the virus into the new host cell. The fragment is, at any rate, inaccessible to deoxyribonuclease or any other informative reagent, but it would be plausible to equate the genetically identified fragment in *Salmonella* phage with the DNA preparations from the pneumococcus. Although we are thus hindered in chemical studies of *Salmonella* transduction, we can console ourselves with the possibility of some understanding of virus biology, and with the merely technical advantages of *Salmonella* for genetic research.

Details of the relation of the genetic fragment with the maturing phage particle were discussed by Zinder earlier in this symposium. To review very briefly, transducing activity has been detected in lysates of *Salmonella* species roughly in proportion to the number of phage particles (Lederberg *et al.*, '51; Zinder, '53; Zinder and Lederberg, '52). Some means of selective isolation is always needed to detect the altered cells, for any given trait is transduced by about one per million phage particles, and the number of phages that can be effectively adsorbed by a single bacterium is limited. The competence, i.e., the range of traits that can be transduced by the various particles in a given phage preparation, is rigidly determined by the genotype of the host cells. Every character that has been tested is subject to transduction, with only second-order differences of efficiency as described by Zinder. These characters include nutrient requirements, sugar fermentations and inhibition, antibiotic resistance, motility, and flagellar antigens. The active material in the lysates is identified with phage (as carrier) not only by surface resemblances in numerical proportionality to plaque count, size (gradocol filtration; sedimentation), tolerance to heat and disinfectants, adsorption on various bacterial serotypes, and neutralization with antiphage serum, but also in the correlation of transduction with virus infection and lysogeny at low ratios of phage:bacterium. This shows best that the same skins enclose phage and fragment. These may be differentiated, however, by the use of ultra-



violet light (the fragment showing a much smaller cross section than the infectivity) or by the use of bacterial hosts to which the phage is not adapted and in which it fails to proliferate. Finally, it should be noted that transduction is mediated in much the same fashion by "temperate" phage grown in the lytic cycle, "temperate" phage obtained by ultraviolet induction of lysogenic bacteria, and "lytic" phage mutants (necessarily grown in the lytic cycle and applied to lysogenic, immune recipients).

### *Lysogenic conversion*

A lysogenic bacterium has been understood to be distinctive in its hereditary makeup since the early investigations of Burnet ('34) and others, but the preconception of most geneticists (including Lederberg, '49a) doubtless favored a cytoplasmic localization of the latent, symbiotic virus. The sexual system of *E. coli* K-12 permitted the first explicit investigation of the genetics of lysogenicity (Lederberg, '51; Lederberg and Lederberg, '53; Appleyard, '53; Wollman, '53) which showed that this trait, far from depending on exceptional cytoplasmic factors, rested on the same basis as the other mutually linked genetic determinants of the bacterium. The penetration of the "temperate" phage lambda into a sensitive host bacterium is thus followed either by bacteriolytic multiplication of the phage, or by the incorporation of the genetic material of the phage into the bacterial chromosome at a specific locus, *Lp*, closely linked to *Gal* (galactose fermentation). This virogenetic locus is reproduced *pari passu* with the remainder of the bacterial genotype. In some of the lysogenic descendants, it may again become autonomous, to reinitiate the bacteriolytic cycle and the release of infective virus. It is not yet clear whether the virogenetic segment simply adheres to the homologous locus or actively replaces it. Some analogies with transduction involving the same phage suggest that both occur in sequence, which may also explain Appleyard's "double lysogenies" ('53).



In either event, should lysogeny be characterized as a species of genetic recombination? To traduce Hershey ('53), the bacteriophage particle can be considered as a miniature, somewhat simplified bacterium with an outer membrane or skin and an internal nucleus (DNA or vegetative phage). When a host bacterium is attacked, the skin and tail of the phage are left behind and the nucleus penetrates (as in the fertilization of egg by sperm) to initiate the developmental cycle of infection or lysogeny which will ultimately result in the reappearance of infective (complete or mature) phage particles. Lysogeny consists of a strikingly intimate union of the phage nucleus with the bacterial genotype. We have the arbitrary choice of defining the lysogenic bacterium (in common with other symbiotic complexes; J. Lederberg, '52) as the association of two organisms, or as a novel recombination having a good deal in common with fertilization or transduction. The recombination frequently results in alterations of bacterial behavior having to do with host-virus interrelations (Luria, '53; Lwoff, '53; Boyd, '54). But it may also result in more insistent changes of bacterial qualities that would not at first sight have been related to a virus: toxin formation in the diphtheria bacillus (Groman, '55), colonial morphology in *B. megatherium* (den Dooren de Jong, '31; Ionesco, '53), and somatic antigen in group E *Salmonella* (Iseki and Sakai, '53; Uetake *et al.*, '55). In this respect, these lysogenic conversions resemble the transduction cited, but the alterations here are inseparable from lysogenicity, i.e., the genetic quality is specifically associated with the phage nucleus, not a desultory companion.

This concept of lysogenicity implies that the incorporated phage nucleus now functions as a segment of a bacterial chromosome (Lwoff, '53). The conversions might even be represented as atavistic remnants of the bacterial functions of such segments before their differentiation. Indeed, the phylogeny of any virus cannot be safely argued, since primary vestige cannot be distinguished from secondary adaptation of the parasite. It therefore cannot be said whether

the virus has evolved from the bacterial segment become suddenly autonomous, or whether the incorporation of the segment is the extreme of parasitic specialization. In fact, we should not be too complacent that the latent virus is always embodied in the bacterial chromosome lest differences be overlooked in other systems that might lead to a broader perspective.

#### TRANSDUCTION AND RECOMBINATION

Genetic transduction may be divided into its initial and terminal phases, the fragmentation of the genetic material, the introduction of fragments into a new cell and their incorporation in the genotype. As far as present information is concerned, the fragmentation is essentially random, although Zinder's data show differences (possibly topographic) of timing in the assumption of different fragments into mature phage. Unfortunately, since nothing is known of the localization of latent virus in *Salmonella*, random assumption cannot be related to what might be an equally indeterminate intrabacterial site of virus fixation or growth. Linked transductions (Ephrussi-Taylor, '51; Hotchkiss and Marmur, '54; Leidy *et al.*, '53; Stocker *et al.*, '53) show that the fragments are not "single genes," but it cannot be said from these experiments whether "crossing over" of linked factors results from initial fragmentation or a later differential implantation (or both). In the DNA-mediated transductions, we can ask whether more gentle preparative methods might preserve otherwise broken associations, but information on possible limitations on the size of effective particles is lacking. But at least in a qualitative way, we can readily visualize how chromosomes can be fragmented without destroying the vitality of the parts: this is a familiar intracellular experience in radiogenetic work. We can also speculate how (or accept the fact that) such fragments are introduced into a new bacterium. But how shall we understand incorporation? A view once stated (Lederberg, '49a) that "from purely mechanical considerations it would seem most likely that the

transforming agents are incorporated into a cytoplasmic system like that of *kappa* . . . a parallelism with induced lysogenicity" was based on the incorrect premise that "the more credible reports uniformly picture the *acquisition* of a genetic function" and the lack of data on factors other than the capsular polysaccharide. Of course transduction may involve any element of the entire genotype and entail the replacement of the homolog, not merely an addition of a genetic factor. This is especially well shown in the substitutions of alternative (multiple) alleles for the flagellar antigens in *Salmonella* (Lederberg and Edwards, '53) which have been carried back and forth repeatedly. After transduction, the allele that had been replaced could not be detected by either phenotypic or genotypic (transductive) analysis. There is, therefore, a problem of integration, not just addition. It is difficult to see how a cytoplasmic system, that is, a genotype as dis-integrated in the living cell as it is in a DNA preparation or a phage lysate, could meet the demands of genetic stability, and even more so to envisage mutual replacement on this scheme.

Fortunately, a new transduction system involving *E. coli* K-12 and lambda (Morse, '54), furnishes some tangible facts to bolster these a priori doubts. This system differs from *Salmonella* insofar as the only genetic factors so far found to be capable of transduction are a cluster of closely linked loci (*Gal*<sub>1</sub>, *Gal*<sub>2</sub>, etc.) concerned with galactose fermentation. This cluster is also linked with *Lp*, the locus of fixation of lambda in the lysogenic bacterium. The second important difference is the persistence of the heterogenic state; that is, the transductions lead to clones that are apparently "heterozygous" for the *Gal* factors involved. The heterogenic bacteria later segregate to give either of the two parental forms (with respect to the *Gal* factors) or, more rarely, crossovers. In this species, therefore, introduction and incorporation are separated in time, and can be more readily analyzed. A given

heterogenic clone permits crossing over between the introduced fragment and the intact genome, with different results in different cells. Sexual recombination analysis, especially the segregation behavior of diploid hybrids, assures that the *Gal* and *Lp* loci are normally integrated (Lederberg and Lederberg, '53) into the linkage system. This provides a partial answer to the previous question on the timing of crossing over in transduction of linked factors, but the incorporation or crossing over of fragments that Muller had visualized ('47) must still be detailed.

Here we face the dilemma of this symposium: Shall we adhere to a mechanical breakage picture, with its obvious difficulties in the postulation of precise double breaks, with the even greater improbability of double crossing over? Or shall we dispose of familiar difficulties by an appeal to the unknown, by postulates of the mechanism of genetic replication? As long as facts and fancies are clearly separated, some speculations may be in order.

Following Belling's lead ('33), we may be strongly tempted by one or another copying-choice principle in connecting crossing over with chromosome reproduction rather than chromosome breakage, as illustrated in figure 1, which begins with the fragment introduced into a new cell. We should not balk then at postulating its synapsis with the homologous element: it is inconceivable that replacement could occur without specific pairing of some sort. The next steps are more obscure, but the end result is an effective double crossover between the fragment and the intact chromosome. Sequence A shows two pairs of breaks, on the mechanical theory. Sequence B shows Belling's theory, with a choice of interconnections after reproduction of the elements; sequence C is very similar, with a choice in the models for reproduction of the new chromonema.

The copying-choice models (B and C) may also be applicable to other enigmatic examples of frequent double exchange within limited regions, such as the fourth chromosome in



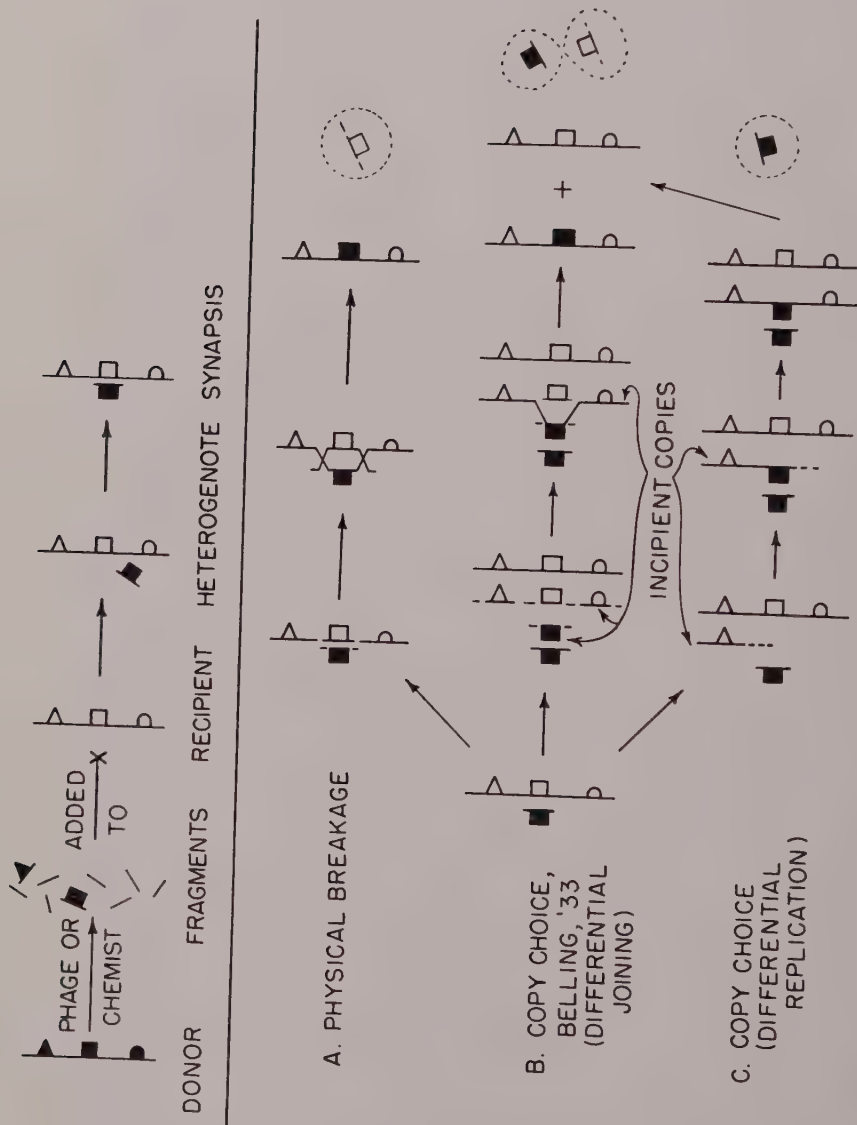


Fig. 1 Hypothetical schemes for the incorporation of transduced fragments. The top line shows the preliminary stages of fragmentation and reintroduction of fragments into recipient cells, common to all three proposals. The fate of the residual fragments, enclosed in dotted circles, is unspecified.

*Drosophila* (Sturtevant, '51), pseudoalleles<sup>2</sup> in *Neurospora* (Giles, '51), maize (Laughnan, '52), and *E. coli* (E. Lederberg, '52), and to the "conversions" in yeast heterozygotes (Lindgren, '53). So far as I know, there are no experimental data on the possible incorporation of small acentric fragments artificially produced at the appropriate stage of meiosis in higher forms, and I would leave to Novitski and McClintock the problem of engineering and interpreting such an experiment. Nor, so far as I know, have intercellular transductions been explicitly attempted with organisms that would be amenable to detailed cytogenetic analysis (some early trials with *Neurospora* auxotrophs were negative or confounded by spontaneous reversion, Ryan and Lederberg, '46).

It is not immediately apparent how these hypotheses can be tested experimentally. Some information might be had from closer study of the immediate progeny of transformed cells, but there are many technical difficulties. To Hotchkiss' account in this symposium one might add that his organism is a *Diplococcus*, and that the units of plating experiments are typically pairs of cells, at least. But this difficulty is not unusual, only more obvious in his organism. Most bacteria have several nuclei within each cell, with the same effect. For further cell lineage studies on genetic replication, mutation, and transduction, it would be indispensable to have a

<sup>2</sup> Alternatively, one can envisage *Ds*-like transpositions from one chromosome to the other along the lines of McClintock's observations ('51). The bearing of *Ds* and other position effects on pseudoallelism (Laughnan, '52) deserves reemphasis, especially where the *cis* and *trans* heterozygotes differ. In maize, the insertion of *Ds* simulates mutation at nearby loci; presumably such insertions need not be all precisely isocal, so that either crossing over, or other means of loss of *Ds* might restore the normal condition in crosses of recurrent mutants. Since gene localization can be studied only with mutations, our concept of a locus as a site of primary genetic function cannot be independently validated, and it may be meaningless to distinguish between a gene and the loci of nearby modifiers. This picture offers no support for the insistence on the origin of pseudoalleles by duplication, or for the notion of structural complexity, separable by crossing over, within genes unless a locus is redefined as a region within which characteristic end effects are generated. Recent reports suggest, moreover, that we shall have to be reconciled to "pseudoalleles" as a feature of any locus that is studied with sufficient diligence.

uninucleate organism whose cytogenetic status could be confirmed by both cytological and genetic means. This would still leave the possibility of confusion from polyteny, a consideration that also arises from other sources described in a later section.

#### SOME RECENT FINDINGS ON SEXUALITY IN *E. COLI*

This subject was comprehensively reviewed three years ago (Lederberg *et al.*, '51). At that time, "*E. coli* K-12 is recorded as a homothallic system, for no preferential compatibilities have been found in recombination experiments involving a wide range of mutants derived from K-12. In particular, no segregation of oppositional compatibility factors could be detected from persistent diploids, in contrast to the . . . mating type mutations in *Schizosaccharomyces pombe*. Preferential compatibility would be very useful for further analysis, and is carefully looked for especially in crosses involving new strains."

Had we waited, it was to have been found among newly isolated fertile strains, but not long after this quotation was recorded, a compatibility system was discovered within the K-12 strain also (Lederberg *et al.*, '52; Cavalli *et al.*, '53). We learned that some sublines of K-12 were compatibility mutants, symbolized  $F^-$ . Crosses of  $F^- \times F^-$  are completely sterile.  $F^+$  (the wild-type state)  $\times F^+$  is fertile, and  $F^+ \times F^-$  even more so. Most of the crosses of previous experiments were  $F^+ \times F^-$ : why was the compatibility system not discovered earlier? It turned out that the progeny of these crosses did not segregate, but were uniformly  $F^+$ , and that mere contact of  $F^+$  with  $F^-$  cells in mixed culture was sufficient to convert the latter to the genetically stable  $F^+$  state. Therefore not until two distant  $F^-$  "mutant" clones had been tried against each other, or an  $F^-$  subline tested for self-compatibility, could the system be detected.

Meanwhile, Hayes ('52) was studying the effect of streptomycin on fertility, and by good fortune, worked with a pair

of stocks that were identifiable as  $F^+$  and  $F^-$ . His discovery of a residual sexual fertility in one of these stocks ( $F^+$ ) after treatments that left a negligible number of viable (colony-forming) cells was therefore promptly related to the compatibility system, and has helped to illuminate it. But I am unable to concur that this experiment speaks for the participation in the sexual process either of "genetic elements extruded by the viable cell which adhere to the cell wall" or of the virus ( $\lambda$ ) inherent in lysogenic strains of K-12. The latter had already been ruled out by the full fertility of nonlysogenic parents (Lederberg, '51), and the former finds no support in the abject failure of the most assiduous efforts to separate subcellular agents that would function in "sexual" recombination (Lederberg, '47a; Atchley, '51; Davis, '50). The streptomycin effect does show at least a physiological distinction between the two parents, and would have an easy explanation if the zygote received most of its cytoplasm from the  $F^-$  parent, and were fertilized without gross contamination by the streptomycin-inhibited substance of the  $F^+$  cell, that is, if mating involved conjugation rather than copulation.

[*Note added June 1954:* Direct support for this picture has recently been obtained by microscopic experiments with very actively mating ( $Hfr$  and  $F^-$ ) cultures, in which one parent is from a motile strain, the other nonmotile. Within an hour of mixing, I find pairs consisting of one cell of each parental line. The pairs are joined laterally and are readily discerned owing to the disparity in motility. After another hour or so, they disjoin. With the micromanipulator, exconjugants have been isolated and permitted to form clones. Usually, both remain viable, and recombinants are found with very high frequency among the progeny of the  $F^-$  cell. It is therefore concluded that the conjugation permits the transfer of a gamete nucleus from the  $Hfr$  to the  $F^-$  cell, followed by karyogamy and meiosis.]

Perhaps the most obscure feature of the K-12 system has been the aberration from mendelian segregation of unselected markers. In the earliest experiments, this was partly ob-



scured by the necessity of selecting certain combinations of markers, usually auxotrophic, in order to detect rare recombinants, but it is equally a feature of crosses where selection can be relaxed owing to the high frequency of recombination. The aberration consists of a relative bias in favor of markers from the  $F^-$  parent. This has led Watson and Hayes ('53) to suggest that the  $F^+$  gamete, which is, according to their version, morphologically subcellular, is also defective with regard to one or more chromosomes. This hypothesis of gametic or prezygotic exclusion is not readily distinguished from the alternative, of postzygotic elimination, by consideration of the haploid recombinants only. The biases would clearly be similar whether the genetic contributions from the  $F^+$  parent were lost before or after the zygote was initiated.

The aberration is seen in the most clear-cut qualitative fashion, however, in the behavior of nondisjunctional diploids (Lederberg, '49b) which occur with highest frequency among the progeny of so-called *Het* mutant stocks. These diploids also show strongly aberrant segregation ratios for markers which are heterozygous, so that this cannot be attributed to prezygotic exclusion. Moreover, they are regularly hemizygous for a pair of linked factors, *Mal* (maltose fermentation) and *S* (streptomycin resistance) though diploid for some fifteen or twenty others. The deficiency for this segment would be sufficient to explain the aberrant segregation, since it should act as a haplolethal and prevent the recovery of any allele linked to it except as coupling is broken by crossing over (Lederberg, '49b; Lederberg *et al.*, '51). Does the deficiency arise by a gametic or a postzygotic process? Closer consideration of the diploid types supports the latter.

When the diploids were first isolated, the hemizygosity was quite perplexing but even more so was the bias with regard to its polarity. In any given cross, most of the diploids were hemizygous for the *Mal* or *S* marker(s) of one parent, but some carried the other. Among the diploids, such a bias could no longer be attributed to linkage to nutritional factors, and no other basic distinction between the parents had been recog-

nized that could account for nonrandom loss of the alternative segments. Nevertheless, since some diploids retained the full set of markers from one parent, and others from the other, the lack of any complete diploids (i.e., heterozygous for *Mal*, *S*) suggested that the elimination occurred regularly after the zygote had formed. Otherwise, one would expect the union of complete gametes to result occasionally in a complete diploid. A few examples of amphitypic diploids, carrying, e.g., the *Mal* allele from one parent, *S* from the other (Lederberg *et al.*, '51, table 6) also suggested that crossing over preceded the elimination.

A more thorough reinvestigation (Nelson and Lederberg, '54) has confirmed this inference. Persistent diploids were isolated from  $F + \times F -$  crosses differing only in their *F* polarity, and tabulated in regard to *Mal* and *S*. Each of the 635 diploids tested was hemizygous for *Mal* and *S*, but regardless of the parental polarity, about four-fifths carried the alleles from the *F -* parent, about 15% from the *F +* parent, and the remainder were amphitypic. Thus the elimination must be postzygotic, but must preferentially involve the segment that had been introduced from the *F +* parent (in the light of later experiments, that is, the migratory nucleus). To account for the incomplete determination, it may be speculated that at meiosis a single locus always breaks on the *F +* chromosome, but that prior crossing over occasionally saves one of or both the *F +* markers with a corresponding loss of their opposite numbers from the *F -* parent.

None of this sounds as if it could be fundamental to a sexual cycle, and if so it might be better to search for more straightforward patterns in other strains of *E. coli*. Some strains at least appear to function independently of the *F +* agent, though they can be "infected" with it. So far, with these strains, such "infection" can be detected only by carrying the agent back into a K-12 line tester stock. Without the good luck of diploid analysis, however, it requires the most tedious development of stocks and study of crosses to study

the basic segregation patterns of new strains, so this is neither the first nor the last time this hope will have been voiced.

Almost no progress has been made on the nature or transmission of the *F* agent. The rapid contagion in mixtures of *F* + and *F* — cells has been mentioned, but this is not paralleled by successful “infection” with cell-free preparations. For example (T. C. Nelson, unpublished), converting mixtures of *F* + and *F* — cells have been poured within a few seconds through membrane filters directly into susceptible *F* — cultures, without the least alteration of the latter. Perhaps, the transmission of the clonally stable *F* + state requires the direct superficial contact of two cells; at any rate, if there is a virological problem at all it may be analogous to the plant viruses which have so far defied artificial transmission.

#### SINGLE CELL PEDIGREES AND TRANSDUCTION IN *SALMONELLA*

For pedigree analysis it is especially rewarding to follow traits that can be determined in single cells. The morphological differentiation observable in living bacteria is so limited that the character of motility stands almost alone for this purpose, but has proved to be most useful. When a nonmotile mutant of *Salmonella* is exposed to appropriate phage lysates, 1 to 10 per million cells can be provoked to give motile clones. Macroscopically, these are readily selected by platings on a soft gelatin-agar (Hiss, 1897; Colquhoun and Kirkpatrick, '32), on which the nonmotile culture is restricted to the site of inoculation, but through which motile bacteria readily swim as they proliferate, to form progressive cloudy swarms. In addition to the conspicuous swarms, however, Stocker *et al.* ('53) also described trails or chains of small colonies that might extend 10 to 20 mm into the agar. We concluded that the trails represented an abortive transduction, whereby a genetic factor was transduced to a nonmotile cell in a form capable of restoring motility to the recipient, but incapable of reproducing with it. The transformed cell would therefore divide to give one motile and one nonmotile daughter. The

former would continue to move, the latter would (rather promptly) stop and, by forming a colony in situ betray the trail of the cell. Since the trails were definitely unbranched, at least in the terminal portions that could be carefully examined, we concluded that the metapoietic particle did not reproduce at all during a hundred or more bacterial generations. Sooner or later, the trails terminated, presumably from some accident; there was no indication of a swarm issuing from a trail. Subsequent micromanipulation experiments both here and in Stocker's laboratory at London have provided a new approach to the problem.

If mixtures of nonmotile bacteria and competent phage are planted in an oil chamber, and examined with the microscope, motile cells begin to appear after about 2 hours' incubation. Unfortunately, as many as two or three divisions may take place during this interval, which truncates and complicates our genealogies. The motile bacteria can, however, be readily trapped when they are permitted to swim into adjoining empty droplets, and thus can be isolated one by one. In the system I have worked on (TM2 —  $\times$  SW-666), the incidence of motile bacteria is rather low, but their viability fairly good. About 10–20% of the isolated bacteria die before engendering sizeable clones. About 5–10% give rise to clones containing anywhere from 25 to 100% of motile cells. The fraction of segregating clones would presumably be higher were it not for the initial bacterial divisions. These motile cells are evidently stable transductions: they engender only motile progeny, and are thus equivalent to swarms. The nonmotile sibs have so far all been parental, none complementary crossovers (with regard to the antigenic factors linked to motility in this transduction), nor has more than one antigenic type been found in a given motile clone.

The remainder of the motile cells are trail equivalents, that is, they give progeny whose motility follows the law of primogeniture, as had been hypothesized from the appearance of the trails in agar. To simplify the following discussion, let us call a cell (or cell lineage) a semiclone if it persistently



transmits motility to just one descendant through several fissions.

The outstanding discrepancy between the microscopic results and inferences from the trails is that a single isolated motile cell may engender during the first five to ten fissions not just one, but up to about 100 semiclones. The discrepancy probably arises from delay in the penetration of motile bacteria into the agar, and their orientation away from the inoculum, until after these early divisions. But after this early interval of apparent "replication" of the motility factor, strict semiclonal behavior is followed until, for reasons unknown, motility is terminated. So far, semiclones have been followed up to 59 fissions, but are usually seen to terminate earlier, often by 20 or 30. The length of time, and the number of progeny involved, have obviously made it impossible to follow any single clone in its entirety ( $2^{59}$  bacteria would weigh 50 tons!) and this picture has been reconstructed from observations on many motile individuals repeatedly reisolated from different clones of different sizes and at various times. Separations of early fissions show, however, that a cell may divide to give one cell a swarm equivalent; its sib the parent of several semiclones. Also, in clones containing large numbers of semiclones, the split during early divisions is grossly unequal: at the 4- or 8-cell stage, one may give 100 semiclones, another less than 10, another none (detected). This rules out any random partition of elements.

How can all this be interpreted? Three hypotheses, which may each have numerous modifications, have been suggested:

(1) The semiclones represent, as originally postulated, the transduction of aborted genes with a limited capacity for irregular replication. This not only fails to account for the sharp transition between the early and later behavior but the *ad hoc* resort to "irregular replication" discourages further study.

(2) The semiclones represent "genes" that are now totally incapable of reproduction owing either to their position or prior accident but still capable of functioning. The multi-

plicity of semiclones represents a degree of polyteny in the bacterial chromosome, and the early divisions serve to distribute the units to the progeny. A cell with but one unit is a semiclon. But what an extraordinary degree of polyteny! And one is surprised that the unit is never incorporated to re-form a motile clone.

(3) The reproductive incompetence of the units is not accidental, but characterized them in the intact donor cell as well. That is, the units are not genes in fact, but the primary products of genes organized in complex bundles. The bundles would perhaps be closely associated with their genic source, but are separated in transduction.

None of the indicated objections to any of these hypotheses is fatal, and we have no certain means of choosing which, if any, is correct. For example (as suggested by Sonneborn), a sterile genetic fragment might be transduced that was still capable of producing the primary products. These units would then initiate the semiclones. Still other hypotheses are imaginable. For the moment, number (3) seems the most fruitful in suggesting further experiments; for example, it attempts to correlate functional status of donor cells with yields of semiclones.

These remarks are presented for two reasons—to elicit further constructive suggestions on interpretation, and to emphasize the value of going back again now from the statistical, populational methodology in microbial genetics to a respect for the individual cell.

#### DISCUSSION

*Chairman HERSHEY:* I should like to bring up again the question that Dr. Lindegren raised this morning; namely, how do you distinguish between linked recombination and transduction in a cross using lysogenicity for one marker and a transducible character for the second? As I understand it, this confusion might appear in the historically important case of *Gal<sub>4</sub>* and the carrier state for lambda in K-12. I think it might be useful if Dr. Lederberg would clear this up.

LEDERBERG: To answer Dr. Lindegren's question first, we would certainly have been confused if transduction had occurred together with sexual recombination. However, the transduction in K-12 involves only a single group of markers concerned with galactose fermentation, and could be neither discovered nor confusing until these were studied. Transduction and sex can be isolated from each other by the proper choice of stocks and conditions. Transduction is mediated by a phage which is readily filterable; the filtrates contain nothing that will function in place of the intact cells in sexual recombination. Also,  $F \times F$  crosses are sexually completely sterile; nevertheless, Mrs. Lederberg has shown that this incompatibility does not hinder transduction by phage, but again, this is limited to *Gal* factors. On the other hand, sexual interaction takes place unhindered though both parents may be nonlysogenic, or if both carry the  $Lp_s^r$  mutation that prevents the adsorption of lambda, although either condition naturally prevents transduction from being effected.

I may add that we have not found deviations from qualitative regularity in segregations from diploids (heterozygous for  $Lp^+Gal^+/Lp^sGal^-$  as well as a host of other markers) that would be called conversion of one chromosome by another. But we lack Dr. Lindegren's advantage of tetrad analysis. It is obvious that incorporation by copying choice could be modified to fit the conversions that he has described, and which deserve the most careful attention.

Dr. Hershey asked about the bearing of transduction on the genetics of lysogenicity. Something was said about this in my talk, but we do not have all the answers yet. However, we still find the clear-cut linkage of *Lp* to *Gal* in crosses where transduction is ruled out as indicated before. In any event, transduction occurs with an efficiency of about one per million phages, which is incomparably lower than the segregation ratios of *Lp* and *Gal*, and would not account for the incidence of both parental couplings in crosses. Since there seems to have been some misconstruction in recent reviews, I want to emphasize that these are quite distinct, though closely linked,

loci and that, while the numerical segregation ratios are strongly biased in  $Hfr \times F-$  or  $F+ \times F-$  crosses, both parental and recombinant classes are found among the progeny. Another useful criterion is the initial heterogenic instability of the transductions; this has never been seen among recombinants. (We are now setting up crosses with the heterogenotes to look for some evidence on the association of the transduced fragment with the homologous segment of the intact genome.)

For a time there was some question about the interpretation of the *Lp-Gal* linkage in the light of the segregational aberrations of  $F+ \times F-$  crosses. I believe this doubt is no longer current; at any rate, there has been no suggestion as to why *Lp* should be "pseudolinked" to *Gal* any more than to any other marker, e.g., *Mal*. But one could even pass over questions on the details of zygote formation and examine diploids heterozygous for these and other markers. The concordant, linked segregation of *Lp*, *Gal*, and all other markers from these diploids is the most compelling evidence of the chromosomal basis of lysogenicity.

ATWOOD: If your semiclones are caused by a nonreplicating product that would stick . . . ?

LEDERBERG: I do not know where they stick. All I know is that they are in the cell. There is no indication as to the localization of the particles that I am talking about here in the bacteria that have been transformed.

ATWOOD: In any case, if they are the result of a nonreplicating product that can function independently of the gene which produces it, then you ought to get semiclones not only following transduction, but also whenever there is a mutation to nonmotility.

LEDERBERG: That is a point I should have made. One should look, in all experiments of this kind, not only for permanent genetic alterations but also phenotypic modifications. That would apply particularly to the pneumococcal and other case provided you had markers where that sort of thing could



be detected. So far, motility is almost the only one that will work, where the phenotype of an individual cell can be diagnosed; and when there is little enough secondary phenotypic delay, as appears to be the case here, not to obscure the results on that basis.

As to whether one should always get this phenomenon, that depends on the hypothetical relation of the products to the gene. It is possible that they are regularly bound to the gene — at least the larger number of them. Then you would get semiclones only under circumstances which would disrupt that relation, namely, in transduction.

However, one or two nonmotile stocks occasionally do give a trail as well as swarms by spontaneous reversion.

But of course even the spontaneous trails could just as well be explained by the other two hypotheses since they could represent cases where the genetic material has been damaged to such an extent that it cannot reproduce.

STENT: It seems to me that if you admit cytoplasmic fusion in the case of recombination, then the role of virus as the possible agent is not entirely excluded.

LEDERBERG: I did not say it was.

STENT: You seemed to think that the *F* agent could not be a virus.

LEDERBERG: I think any group of medical bacteriologists would have slaughtered me if I had tried to give this kind of evidence for the existence of a virus. We should be as careful in defining a virus in this area as in others. We have a contagion phenomenon and would like to find a virus to explain it, since we have no other way to do it, but it has not been found.

STENT: Are no lysogenic viruses known that could identify the virus as acting like the *F* agent? Since transduction is known to be a phenomenon that can occur with virus, then indeed under the concession that the *F* agent is a virus, an understanding of the phenomenon would be advanced. At least it would be unified.

LEDERBERG: Our understanding of the phenomenon will be most advanced when the  $F$  agent is isolated from the cells so that its genetic properties can be described.

STENT: But it cannot be isolated because, if the  $F$  agent is defective prophage that never achieves maturity, i.e., exists only in the cells in the vegetative form, then any attempt to break up the cell would immediately destroy it.

LEDERBERG: It does get over from one cell to another.

STENT: Yes, through cytoplasmic fusion.

LEDERBERG: I think we are now talking about words. I would describe such an element as the gamete of the bacteria, and would then proceed to do experiments to determine the genetic content of that gamete; those that have been done indicate that the zygote that is formed is complete. If you want to attribute virus-like properties to these agents, too, then you must think of experiments to settle the problem. But I think that, until it is isolated, we had better be careful about assuming it is a virus, because the impetus for trying to isolate it might be lost. I consider that to be the most important question in that particular area. The results have been so uniformly negative that we might well be suspicious of that negativity and begin trying to think of things other than viruses to explain it.

It is quite conceivable that in a situation where a virus-like agent — or call it an  $F^+$  agent — is necessary for the effective contact between, say an  $F^+$  and an  $F^-$  cell, that agent may be what bores the hole in the  $F^-$  cell. There are any number of possibilities. But in this part of the story where there are some facts, I preferred to stick to them.

In spite of the temptation to speculate, the facts are that, in order to get a mating, one of the parents must be  $F^+$ , and presumably a surface property of the bacterium is altered. However, the  $F^+$  agent, this thing that is capable of converting, is not by itself a sufficient condition for the  $F^+$  property of compatibility. It is not even a necessary condition since there are compatible cultures ( $Hfr$ ) that cannot convert. Aeration of an  $F^+$  culture produces a population of cells,

every one of which has that agent, because they can give rise to clones which are capable of converting, but none of which have the property of genetic recombination.

This is why I have not wanted to be too specific too soon about the possible effect of the  $F^+$  agent, and prefer to say that it has an effect on the surface of the cell rather than to say that it itself is the character. We are really back to the old story of the relation of gene and character. Here is at least one criterion by which they can be separated; namely, this aeration where there is still the heredity of  $F^+$ , but not its action, in those cells.

STENT: I think the distinction perhaps is more than words because it would explain the streptomycin effect, which I understand is unexplained under your conception.

LEDERBERG: You probably misunderstood my discussion on that point. The streptomycin effect, as we now understand it, does strongly suggest that the cytoplasm of the zygote does not receive a very large contribution from the cytoplasm of the cell that has been steeped in streptomycin, but that is all that one needs to postulate.

On the notion of a conjugational type of exchange of nuclei and a limited amount of cytoplasm, too, if you like — we have no criterion for it — that problem is completely solved.

STENT: Why does treatment of the  $F^-$  cell with streptomycin cause infertility?

LEDERBERG: For the reason that I have just indicated, that on this notion the larger part of the cytoplasm of the zygote is derived from the cytoplasm of the  $F^-$  cell. If that cytoplasm is loaded with streptomycin, that cell is incapable of further development. If there are ways of removing the streptomycin or of inactivating it, there might be further development.

It should be pointed out that the viable counts that are gotten on cultures so-called killed with streptomycin are extremely variable, depending on the details of the conditions of plating, which shows in a way that one has to be very careful in speaking of cells that are “killed” in this particular

province. One should speak quite specifically of which functions are reversibly, and which are irreversibly, inhibited at that time.

I am trying to narrow down what the experiment shows. You can offer one specific hypothesis to explain it; I can offer some more. But in order not to enumerate hypotheses at great length, one can make the generalization that the experiment does indicate that the larger part of the cell substance of the zygote does come from the *F* — cell. But that is equally compatible with quite a range of hypotheses about what it is that the *F* + cell contributes, so long as that does not include a lot of cytoplasm containing streptomycin.

PLOUGH: I have a question on a point which both you and Dr. Zinder mentioned. In your recently published studies with Stocker and Zinder on transduction of *Salmonella* antigens, you found that, ordinarily, it was not the specific flagellar antigen of the donor strain which was transduced, but rather the filterable agent (FA) induced the reappearance of the flagellar antigen which the recipient strain presumably had originally. This appears to me to be more easily explainable as a reversion caused by a general mutagenic action of FA.

LEDERBERG: This was ruled out by comparing the effectiveness of phage that had been grown on the nonmotile recipient with phage grown on other motile or nonmotile strains, or to be sure that *strain* homology was not involved, on rare spontaneous motile reversions when these could be obtained. In no case did the phage grown on a given nonmotile indicator confer motility upon it, whereas the other phage preparations were almost always effective. By testing different nonmotile strains against one another, seven distinct groups, presumably different mutant loci, were identified that involve flagellar formation; two which affect their ability to function if formed, and two concerned with their antigenic content. The fact that motile transductions usually gave bacteria that had restored their innate antigenic potentiality simply means that different genetic factors determine whether flagella should be formed



at all, and what their antigenic potentialities would be. This would be, by the way, an almost trivial instance of multigenic control of an antigen, except that we can identify the organelle that underlies the antigen. I did not have the time to go into the dynamics of phase variation; it fits in very nicely with the product story.

HOTCHKISS: I agree with Dr. Lederberg that it is going to be very important to study individual pedigrees in transformed populations. My remarks in this symposium show that we have already started along that line. We have also been using streptomycin resistance to select, immediately within the same hour that DNA was added, cells which have been destined to be changed by the DNA. These were spread on agar and at various times the segregation of the streptomycin resistance and factors linked to it were studied.

As to nomenclature, I think it would be well if we pointed out explicitly that the word "transformation" has disadvantages since it comes from general usage and is adapted for a rather specific sense. But it does have historical value. Many people know what bacterial transformation means. Therefore, I should like to recommend that we retain "transformation" as the generic term, and save "transduction" for the phage-mediated transformations. It seems inadvisable to use the term "transforming principle" except when talking about an abstract principle, rather than an actual material. The term "transforming agent" can be used in reference to a material entity; if it is a phage, it becomes a transducing agent.

LEDERBERG: No one can deny that in all these experiments cells are being transformed, or rather their properties are being altered. In that context, there is no objection at all.

HOTCHKISS: Also, one may have a transformed cell, or "transformant," while you have defined transduction so that it is only the character which is transduced.

LEDERBERG: Precisely. I hope I kept that straight: the transduction of a character from cell A to cell B which results

in the transformation of cell A to type A. I see no reason at all for not using both terms for what they do mean, as the occasion demands. For the same reasons you have indicated, that "transformation" was so vague (meaning, essentially, change) it has been applied to several phenomena with no implication of genetic transfer, even by Griffith himself, in the change from smooth to rough. There is also the transformation of vegetative phage into mature phage, and so on. Perhaps another term is needed to distinguish phage-mediated transductions (or transformations), though perhaps we ought to learn a little more about them first. But "a man coins not a new word without some peril and less fruit; for if it happen to be received, the praise is but moderate; if refused, the scorn is assured" (Ben Jonson).

BERTANI: There seems to be complete similarity of behavior between the transforming agent in your "semiclones" and the phage superinfecting lysogenic cells. Such phage enters the cells (which are carrying a genetically related prophage), but does not affect their ability to grow. As the cells divide, the superinfecting phage does not multiply or multiplies very little, and it is thus diluted out among the growing cells. This state of the superinfecting phage has been called "preprophage." The preprophage, like the transforming agent in the "semiclones," can be considered physiologically active, because, if the cell that carries it lyses, phages of both the prophage and the preprophage types are liberated. The preprophage, also like the transforming agent, has a small chance of "transforming" the cell that carries it, by substituting its own type for the prophage type ("prophage substitution"). When several phage markers are present, this process can be shown to be a true genetic recombination between the prophage and the preprophage.

LEDERBERG: Then the similarity is not complete. Stable transformations are not found out of semiclones. The semiclones occur in clusters from a single parent bacterium.

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# BIOLOGICAL CONSEQUENCES OF THE COMPLEMENTARY STRUCTURE OF DNA

(ABSTRACT)

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Although the chemical formula of DNA has suggested that the basic structure is that of a very long, unbranched polynucleotide chain, recent X-ray diffraction evidence (Wilkins *et al.*, '53; Franklin and Gosling, '53; Watson and Crick, '53a) reveals that the fundamental stereochemical unit contains two helically intertwined chains. The chains are joined together by hydrogen bonds between pairs of bases, a single base in one chain being hydrogen bonded to a single base from the other. The bonding scheme is highly specific; not only must one member be a purine and the other a pyrimidine, but adenine must pair with thymine and guanine with cytosine. A given nucleotide can occur on either chain, but when it does, its partner on the other chain is specifically determined. This results in a complementary relation between the sequence of bases on the two chains. The sequence of bases on one chain is believed to be irregular.

The presence of complementary strands suggests a self-replicating mechanism since if the two chains can unwind and separate, then each can serve as a template for the formation of its complement (Watson and Crick, '53b). If this is true, we might expect DNA to be metabolically a very stable substance and to show very little turnover as measured by isotopic techniques. In other words, we should expect the DNA molecules to be specifically conserved during replication. Unfortunately, the present experiments do not yield a simple answer. While most investigators have suggested a very slow



DNA turnover, Daoust and his collaborators ('54) using mammalian tissue have interpreted their recent results as indicating a complete replacement of the parental DNA  $P^{32}$  atoms during replication. It appears possible, however, to provide alternative interpretations of their data in which the parental DNA does not turn over. Likewise, the isotopic experiments involving bacterial viruses do not provide a result capable of an unambiguous interpretation. Whereas it is generally concluded that approximately 50% of the parental DNA is transferred to progeny particles (Kosloff, '53), no convincing evidence has been obtained as to the nature of the transfer. The important point as to whether the transfer is in the form of genetically specific DNA molecules or at the level of non-specific nucleotides has not yet been resolved.

The DNA replicating scheme does not provide a mechanism for separating the two intertwined chains. Although we have suggested (Watson and Crick, '53b) that this may be accomplished by untwisting, the very large number of turns necessary to achieve complete separation suggests that alternative explorations should be considered. In a recent proposal by Delbrück ('54), untwisting is circumvented by the production of systematic breaks along the parental chains, followed by reunion of parental strands with the growing ends of the newly formed strands. Although at present the scheme must be regarded as purely theoretical, it has the advantage of providing the eventually testable prediction, that following replication, the parental atoms will become equally distributed between parental and progeny strands.

#### DISCUSSION

STENT: I should like to report briefly an experiment relevant to the DNA duplication scheme proposed by Dr. Watson, carried out by Dr. Itaru Watanabe in our laboratory, in collaboration with Dr. H. K. Schachman. For this experiment, bacterial cells were infected with T2 phages whose DNA had been labeled with  $P^{32}$ . At various stages after infection, the infected bacteria were exploded by decompression and the

sedimentation characteristics of the radiophosphorus of the resulting lysate examined in an ultracentrifuge separation cell. We focused our attention on five arbitrary fractions, represented by the following sedimentation constants in Svedberg units,  $S$ : Fraction I =  $0.5S$ , fraction II =  $10S$ , fraction III =  $100S$ , fraction IV =  $800S$ , fraction V =  $3000S$ . Fraction I corresponds to  $P^{32}$  contained in low-molecular-weight material, fraction II to that forming part of DNA fibers. Fraction IV represents  $P^{32}$  in intact phage particles, while fraction III would contain any  $P^{32}$  introduced by the parental phage which now forms part of structures with sedimentation velocities intermediate to those of fibrous DNA and mature bacteriophages. Fraction V, finally, would represent parental  $P^{32}$  attached to bacterial constituents larger than intact phage particles. At the same time, the amounts of  $P^{32}$  of the lysate soluble in TCA (fraction A), soluble in TCA only after treatment with DNase (fraction B), and insoluble in TCA even after DNase treatment (fraction C) were determined.

The results of this experiment are shown in form of a bar graph (fig. 1), in which  $t$  indicates the number of minutes elapsed between infection and disruption of the bacterial cells, and in which the percentage values of the ordinate indicate the amount of  $P^{32}$  in each of the arbitrary fractions I, II, III, IV, V or A, B, C at different stages of the infection. When the cells are broken open immediately after infection — when the DNA of the infecting phage has just penetrated into the cell — about 60% of the parental phosphorus appears as fraction II, the fraction sedimenting just like DNA fibers. A similar percentage is found as fraction B, i.e., is DNase sensitive. Hence one may feel confident that fraction II represents DNA. The rest of the  $P^{32}$  is distributed about evenly among the other fractions. At later times, we find that fractions I and A increase, although fractions II and B retain at least half of all the parental  $P^{32}$  until the fifteenth minute. At that moment, mature progeny phages begin to make their intracellular appearance, containing the transferred parental  $P^{32}$  now appearing as fractions IV and C. Fraction III at

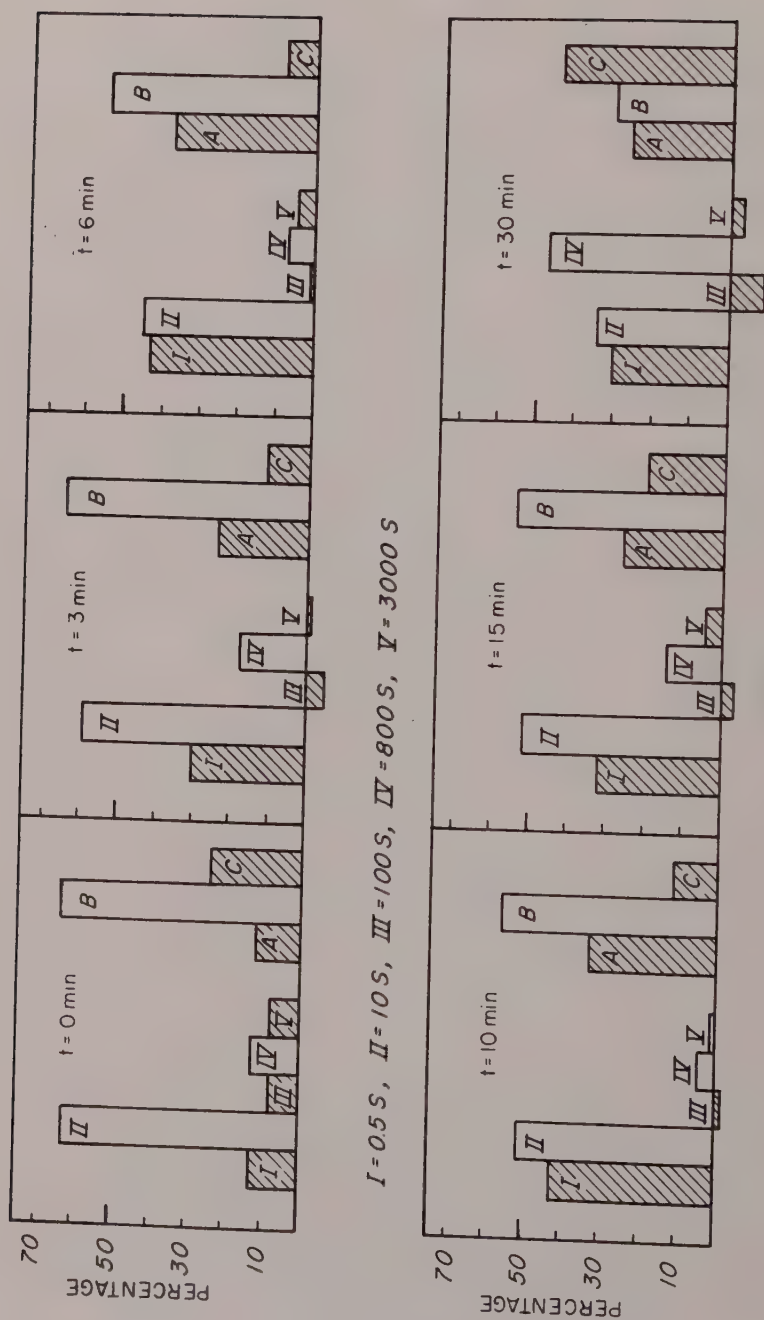


Figure 1

no time contains any appreciable amount of the  $P^{32}$ , indicating that the parental DNA has not formed any stable union with any cellular constituent with sedimentation properties in this range. Nor do there appear to exist phage "precursors" intermediate in size to molecular DNA and mature phages which contain any DNA and are stable to the method of cellular disruption employed.

It is thus seen that on one hand an appreciable fraction of the parental DNA appears to be broken down to small-molecular-weight substances in the course of its duplication. On the other hand, an even larger fraction does remain in the form of high-molecular-weight DNA. The mechanism of DNA replication proposed by Dr. Watson would appear to demand the conservation of the macromolecular nature of the parental DNA molecules and is therefore not incompatible with these findings.

HERSHEY: Dr. Watson has mentioned the important question of whether atoms of the parental viral DNA are conserved in functionally intact structures during multiplication. This question is related to the more general one concerning turnover of DNA that has already been discussed rather extensively in this meeting. I want to add three bits of information — briefly, because we are not going to decide these questions today.

First, replacement of atoms in bacterial DNA cannot be detected during multiplication of cells. If we start with  $C^{14}$ -labeled cells, then let them multiply for six generations in a medium containing specific nonradioactive precursors of DNA, we find that the  $C^{14}$  in each of the individual DNA purines and pyrimidines has been perfectly conserved.

Second, the identical experiment can be performed by observing the multiplication of  $C^{14}$ -labeled bacteriophage. Here, too, we find that the progeny bases are labeled in the same ratio one to another as they were in the parental DNA, though the conservation in this instance is only about 50%. This retention likewise is not influenced by competitors.



Third, if you look for a correlation between the transfer of genes and the transfer of atoms from phage particle to phage particle, the situation is by no means as discouraging as it used to be. The early experiments had seemed to show that irradiated virus could contribute atoms but not genes to the progeny of unirradiated virus when the two were present together in the same cell. I am not prepared to deny this possibility, but I can say that it has not been proved. Garen and I have been reinvestigating this question very assiduously and all we have learned is that the technical problems are difficult but not insurmountable.

COHEN: I have not read the Leblond paper and so am in a poor position to comment on it. I did hear some discussion of it at the Federation in Atlantic City, and in particular I did hear at least one paper describing a similar type of experiment in which the conclusions run directly contrary. I believe there are others in the literature, too. But the one which I heard, by A. D. Barton of the McArdle Laboratory, seems to be what you are looking for.

The experiment involved partial hepatectomy — we are talking about mammalian tissue, which seems to be the stumbling block at the moment — following which two kinds of labels were administered — glycine-2-C<sup>14</sup> and radiophosphorus; the animals were kept for periods up to 75 days after this partial hepatectomy and then partially hepatectomized again, removing the other lobes, and then allowed to regenerate for 4 days.

Examination of the regenerated lobes after the second operation indicated that both labels in DNA were retained throughout the 75 days after the first operation (ten times the specific activity of the RNA or acid-soluble fraction) and throughout 2–4 days of the second regeneration decreased only slightly, in spite of the synthesis of poorly labeled DNA, which would seem to indicate that the levels were retained.

HOTCHKISS: I think I should like to return to the question of the Stevens and Leblond experiments, because there is almost no audience in the world for which there is a more in-

teresting question. They made assays of various tissues, counting the number of cells that were in a mitotic stage. I believe this was done as well as it could be done. They then measured incorporation of new  $P^{32}$  into these tissues with these known mitotic rates and calculated, from cold tissue picking up hot atoms coming from a certain background or pool of phosphorus, the amount of DNA made in proportion to the number of mitoses. The calculations seemed to indicate that twice as much DNA was synthesized as was needed; that is, enough for two new cells for each mitotic division. This involved the assumption that the amount of incorporation was referable to only those cells which were found in mitotic state. If you think a moment, you will realize that the assumption that you can refer this incorporation to those cells only is based on the essentially qualitative observation that incorporation is occurring *mainly* in cells undergoing mitosis. But it had never been shown that there was no incorporation at all in places where there was no mitosis. Therefore it is hazardous to relate incorporation only to dividing cells. The direction in which these investigators worked, going from unlabeled to labeled tissue, was the one which makes experimental assay very difficult.

Secondly, of course, they were working with a difficult kind of tissue; but we all want to know about this, so we will excuse them for working with animal tissue.

The other kind of difficulty the authors had was in knowing what the isotope pool was from which to estimate the actual amount of incorporation; there was only a single administration of isotope, and a changing pool, starting high and becoming low. They had to make an assumption about the average state of that pool, and did so in an unsatisfactory way.

Finally, they had to estimate the amount of DNA in the tissue. This they did by a common method which was in very frequent use up to about 2 years ago, but has been shown by Davidson and Smellie to give impure products. This is particularly critical for this kind of investigation, since the DNA

is the slowest component of the tissue to accept isotope, and is the most likely to be grossly contaminated by other  $P^{32}$ -containing substances.

So I feel that, although these workers have presented an exciting claim, we should be very skeptical of it because of the great difficulties in operating in the particular way they did; and at the moment we should give all our credence to such experiments as have just been described by Dr. Hershey, operated in the other way where one measures actually the disappearance of the isotope.

Stevens and Leblond therefore did not actually show destruction of the preexisting DNA, they merely estimated that incorporation so measured with all these difficulties was more than expected.

GALL: I was wondering if there was any reason to suppose that a similar model could or could not apply to the RNA molecules. I have in mind particularly some of the viruses containing RNA.

WATSON: Since last fall, I have been working at Cal Tech with Alex Rich on RNA structure. We have found that oriented fibers can be drawn from highly polymerized RNA preparations and that these fibers yield X-ray fiber diagrams. Although these diagrams are not nearly so good as the corresponding DNA photographs, they do establish that all RNA's, no matter the source, give the same X-ray pattern. A common RNA structure thus exists. However, up till now, we have been unable to interpret the RNA diagram and we can only state that a similarity (perhaps superficial) exists between the RNA and DNA diagrams.

The base ratios in RNA offer a perplexing picture. In RNA's from a large number of diverse sources, an equivalence of adenine with uracil (thymine minus a methyl group) and guanine with cytosine is observed. This happens often enough to make us believe that it has a fundamental basis. In the plant viruses, however, you definitely do not find the base pairs; and it is in the plant viruses that one might expect

to get the best RNA. So if the structure is like DNA, we have to devise a mechanism so we can get away from base pairing. That we have not done.

COHN: I want to extend the remarks I made a moment ago and corroborate what Dr. Hotchkiss has said. The question of what molecules are actually used to make DNA (or RNA, for that matter) is still unanswered. The experiments that have been reported, e.g., the Leblond type of experiment, involve assumptions as to what are precursors. There are actually two assumptions to make. The first is the time course of a particular pool, and the second is that you know what the proper pool is.

The first of these cannot easily be assayed quantitatively, and the second is very difficult to assay even qualitatively. I think under the present circumstances, with our lack of knowledge of immediate precursors, we had best put our faith in the retention type of experiment which I mentioned and which Dr. Hershey spoke of also, rather than the incorporation type, because the incorporation type rests on calculations based on two very shaky premises.

I think that for corroboration of this, one has only to talk to the people who are trying to find out what the precursors are. For example, from Brown's laboratory at the Sloane-Kettering Institute and in Potter's at Wisconsin we are finding out what a sorry state that phase of the problem is in; and without a knowledge of what the precursors are, the correct specific activity calculations cannot be applied.

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# TETRAADS AND CROSSING OVER

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SEVEN FIGURES

Genetic recombination is now being investigated in forms ranging from viruses and bacteria to *Drosophila* and maize. Between these extremes there are organisms almost uniquely suited for studying the details of meiotic crossing over — those lower plants in which tetrad analysis is feasible. In the most promising of these organisms, mapping is rapidly reaching the point where they can make important contributions. This may therefore be an appropriate time to appraise what information tetrads have contributed so far regarding the mechanism of crossing over, to evaluate what they are potentially capable of revealing, and to examine the status of some specific organisms that are especially well suited to provide tetrad data in the future.

## INFORMATION FROM TETRAADS

The most important advantage of tetrads over single strands is that the positions and types of all exchanges can be determined precisely. This is essential if the effects of chiasma interference and of chromatid interference are to be distinguished from one another. [In this respect, tetrads also possess a distinct advantage over half-tetrads, such as attached-X chromosomes, where information is incomplete even for those exchanges that can be detected. Other advantages of tetrads have been enumerated by Papazian ('52), Perkins ('53), and Barratt *et al.* ('54).]

The ability to detect all exchanges gives tetrads a unique advantage for experiments designed to describe crossing over

and interference in many chromosome regions simultaneously. So far they have not been used to any extent for these purposes. However, considerable tetrad data have been obtained on exchange types and frequencies in a few regions at a time. These are chiefly of interest for the information they provide on chromatid interference.

*Chromatid interference.* Since information on chromatid interference is essential for an understanding of crossing over, the main experimental results will be briefly examined.

Table 1 shows Lindegren and Lindegren's 1942 data for adjacent pairs of exchanges in *Neurospora*. Chromatid interference is indicated by striking departures from a ratio of 1:2:1 for 2-, 3-, and 4-strand double-exchange types. However, an alternative explanation exists. The Lindegrens pointed out that the excess 2-strand double exchanges in regions II and III, across the centromere, could have originated by abnormal centromere assortment rather than by crossing over. If the data are retabulated on the assumption that all the twenty apparent 2-strand pairs of exchanges in regions II and III resulted from misassortment of centromeres, the evidence for chromatid interference is greatly reduced, as shown in the lower half of table 1. The Lindegrens considered this second hypothesis and rejected it, but for a reason which I consider not to be adequate.

According to the second hypothesis, centromeres were misassorted in about 1% of asci. (This could occur from equational separation at anaphase I, from spindle overlap, or from ascospore rearrangement.) All the segregation types predicted on this basis are found in the total data, and their numbers agree with expectation, except that misassortment must be assumed to occur more frequently in double-exchange bivalents than in others in order to account for observed frequencies of apparent quadruples. Lindegren and Lindegren ('42) rejected the centromere explanation because they could see no *a priori* reason why abnormal centromere behavior should occur coincidentally with crossing over. The interference hypothesis is subject to exactly the same objection: no *a priori* reason is evident why 2-strand double exchanges across the centromere should occur coincidentally with crossing over in neighboring regions.

TABLE 1

*Chromatid interference data**Neurospora crassa*: 1575 tetrads from Lindgren and Lindgren, '42 (including 1937 data)

sex 4 gap 3.5 3.5 crisp 8 pale

		I	II	III	IV	RATIO (2's:4's):3's
INTERVALS		OBSERVED NUMBERS OF ADJACENT DOUBLE-EXCHANGE TYPES				
		2-strand	3-strand	4-strand	2- or 4-strand	
OBSERVED:	Within arms	I-II III-IV	1 0	2 9	3 5	6 1
	Total		1	11	8	7
	Between arms, symmetrical	II-III I-IV	20 15	3 7	1 6	
	Total		35	10	7	
	Between arms, asymmetrical	I-III II-IV	2 0	4 0	1 5	
RECOMPUTED <sup>a</sup> :	Total		2	4	6	
	Grand total		38	25	21	7
	Within arms	I-II III-IV	1 0	1 3	3 5	
	Total		1	4	8	
	Between arms, symmetrical	II-III I-IV	0 15	3 12	1 6	
RECOMPUTED <sup>a</sup> :	Total		15	15	7	
	Between arms, asymmetrical	I-III II-IV	2 0	4 0	1 5	
	Total		2	4	6	
	Grand total		18	23	21	

<sup>a</sup> These numbers were obtained by assuming that all 20 of the apparent 2-strand pairs of exchanges in II-III, table 1 (Lindgren and Lindgren, '42) resulted from abnormal centromere assortment rather than from crossing over.



Whitehouse's objections ('42) against attributing excess II-III 2-strand pairs of exchanges to spindle overlap, in the Lindgrens' 1937 data, do not apply to centromere misassortment in the total data.

A second group of *Neurospora* data have been analyzed for chromatid interference in table 2. The numbers are too small to be useful unless results from different intervals and experiments are pooled. Two-strand doubles across the centromere are again in excess.

Results from organisms other than *Neurospora crassa* are gathered in table 3. *Neurospora sitophila*, in contrast to *N. crassa*, shows an excess of 3-strand doubles across the centromere. Double exchanges in nearly 400 *Sphaerocarpus* tetrads provide no indication of chromatid interference, but the intervals are so long that its existence could easily have been obscured. Results from thirty-five tetrads of the moss, *Funaria*, are also shown. If we assume, tentatively, that crossing over took place at the 4-strand stage, the maximum ratio of 3-strand to 4-strand doubles in this experiment is 0:19. (Wettstein's results suggest crossing over at the 2-strand stage. More than two types of products were never found among the four members of any tetrad, although recombination occurred in one or more regions of 13 tetrads that were marked at four linked loci.) Results from *Drosophila* attached-X heterozygotes are also given in table 3 for comparison with the tetrad data.

No attempt will be made here to evaluate the *Drosophila* results critically, or to review other types of data on chromatid interference than those from genetically analyzed tetrads. Such a review would include cytological data regarding strand relations in normal chiasma pairs (e.g., Huskins and Newcombe, '41) and data on the genetic and cytological results of crossing over in structural heterozygotes (e.g., Sturtevant and Beadle, '36; Giles, '44; Rhoades and Dempsey, '53) and in ring chromosomes (e.g., Morgan, '33). Cases of recombination greater than 50% would also be pertinent. (But see Michie and Wallace, '53; and Catcheside, Michie, and Wallace, '53, for interpretations of the latter that do not involve chromatid interference.)

The tabulated results include most or all of the adequate genetic tetrad data now available regarding chromatid inter-

TABLE 2

*Chromatid interference data*

*Neurospora crassa*: all multiple-point crosses with established gene order, from published sources other than Lindgren and Lindgren, '42

INTERVALS	OBSERVED NUMBERS OF ADJACENT DOUBLE-EXCHANGE TYPES										SOURCE
	Within arms				Between arms				RATIO (2' 8+4' 8) : 3's		
	2- strand	3- strand	4- strand	2- or 4- strand	2- strand	3- strand	4- strand	Within arms	Between arms		
2 and 3 intervals, 3.5 to 8 units long	1	2	1	3	4	1	0	5:2	4:1	Lindgren, '36	
3 intervals, 8 to 19 units long	5	17	2	15	..	..	..	22:17	..	Lindgren and Lindgren, '39	
Pooled data from 7 multiple-point crosses. Intervals 1.5 to 27 units long	3	14	2	7	9	7	5	12:14	14:7	Barratt <i>et al.</i> , '54 compilation (tables 17 and 18)	
Sex-centromere- 38701-15300. Intervals 1.5 to 25 units long	..	2	..	..	1	1	1	0:2	2:1	Houlahan <i>et al.</i> , '49	
Total	9	35	5	25	14	9	6	39:35	20:9	( $p=0.07$ )	
					(p=0.01)						

TABLE 3

*Chromatid interference data*Tetrads and part tetrads from organisms other than *Neurospora crassa*

ORGANISM	INTERVALS	OBSERVED NUMBERS OF ADJACENT DOUBLE-EXCHANGE TYPES			RATIO (2's+4's):3's	SOURCE
		2-strand	3-strand	4-strand	2- or 4-strand	
<i>Neurospora sitophila</i> (Regions 21 and 29 units long)	Between arms	8	28	8	.. ( $p=0.19$ )	16:28 Wilker, '35
<i>Sphacrocarpus donnellii</i> (Several crosses, regions 2-64 units long)	Within arms	..	33	..	31	31:33
	Between arms	79	147	91	..	170:147
	Total	79	180	91	31	201:180 Knapp, '37
<i>Funaria hygrometrica</i> (35 tetrads, 4-point cross, regions 11-28 units long)	Within intervals (if crossing over is at 4-strand stage)	Unde- tect- able	0	19	.. ( $p<0.001$ )	Wetstein, '23
<i>Drosophila melanogaster</i> (Attached-X chromosome half-tetrads)		2-strand + $\frac{1}{2}$ of 3-strand		4-strand + $\frac{1}{2}$ of 3-strand		
		152		121	121 ( $p=0.07$ )	Beadle and Emerson, '35 (including '33 data)
		7		116	116 ( $p=<0.001$ )	Bonnier and Nordenskiöld, '37

ference. They suggest that such interference does occur, at least sporadically, but the most striking deviations can be explained in terms other than chromatid interference. It is clear that this very important feature of crossing over is still far from being adequately described.

Even if the results of chromatid interference were completely known, their interpretation would depend on the occurrence of sister-strand crossing over. Tetrads can also provide information regarding this second fundamental question.

*Sister-strand crossing over.* In attached-X chromosomes of *Drosophila*, recessive homozygosis frequencies exceeding one-sixth have been interpreted to mean that sister-strand exchanges do not participate in chiasma interference on an equal basis with nonsister exchanges (Weinstein, '33; Beadle and Emerson, '35). In tetrads, the occurrence of second-division segregations in excess of two-thirds provides additional evidence for the same conclusion.

Before examining the data, it may be useful to compare tetrads with attached-X chromosomes (fig. 1). Attached-X's can be thought of as incomplete, ordered tetrads, and are equivalent to two strands taken one from the top half and one from the bottom half of a *Neurospora* ascus. Single exchanges (and a proportion of multiples) result in second-division segregation of alleles distal to the exchange. One-fourth of the possible combinations of two strands taken from top and bottom halves of second-division segregation asci consist of two recessives, and recessive homozygosis frequencies are thus exactly equivalent to one-fourth of the corresponding second-division segregation frequencies. Consequently, second-division segregations exceeding two-thirds are equivalent to recessive homozygosis frequencies exceeding one-sixth.

A gene-centromere interval is measured when second-division segregation frequencies are scored. It can be seen from figure 1 that the equivalent metric for a gene-gene interval is the frequency of tetratype tetrads — those in which the four products are all different ( $AB + Ab + aB + ab$ ).



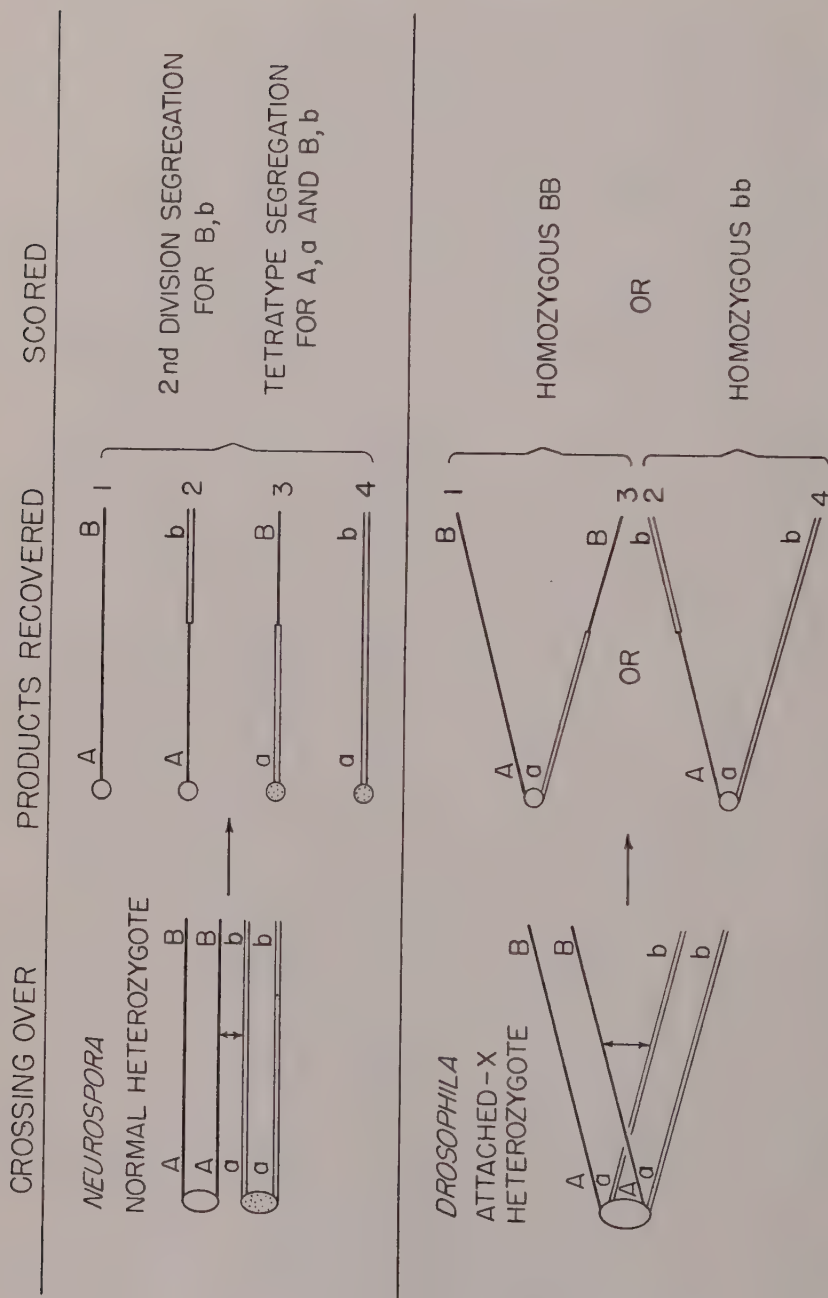


Fig. 1 Consequences of crossing over compared for tetrads and for attached-X chromosomes. A nonreciprocal exchange is shown; reciprocal exchanges would result in heterozygous attached-X's.

Cases where either second-division or tetratype segregations exceed two-thirds are tabulated in table 4. Significant excesses have been reported for segregations in at least eight organisms. (I am indebted to Dr. D. C. Hawthorne of the University of Washington and to Dr. J. N. Hartshorne of Manchester University for permission to use unpublished data.)

For the present purpose, it is irrelevant whether genes are linked, because in order for two unlinked genes to produce more than two-thirds tetratypes, one or the other of them must undergo second-division segregation with a frequency exceeding two-thirds (see Whitehouse, '49, p. 231). However, a high frequency of tetratypes could conceivably result for unlinked genes, independently of crossing over, if centromeres were usually postreduced for one bivalent and prereduced for another.

Likewise, second-division segregations exceeding two-thirds in frequency could result without crossing over if centromeres were postreduced.

Some maximum homozygosis values from *Drosophila* are also shown in table 4, where observed frequencies have been multiplied by four so as to be more easily compared with the tetrad frequencies.

The tetrad results extend and reinforce previous conclusions from *Drosophila*, that sister-strand exchanges do not occur on an equal basis with nonsister exchanges. These results do not, of course, preclude the occurrence of sister-strand crossing over on some other basis.

*Consequences of sister-strand crossing over for interpreting chromatid interference data.* So long as sister-strand crossing over on any basis whatever is not ruled out, it is important to consider what effect it might have on the manifestation of chromatid interference. Schwartz has recently pointed out ('53b) that if exchanges between sisters occurred either at or after the time of nonsister crossing over, they could drastically alter the ratios of double-exchange types that emerged in tetrads. If sufficiently frequent (Sax, '32), they could produce a completely random distribution of emerging types, and hide an actual chromatid interference. I can think of no likely mechanism, however, by which they could accom-

TABLE 4

Tetraploid or second-division segregation frequencies greater than two-thirds and homozygosis frequencies greater than one-sixth

ORGANISM	LOCI	OBSERVED TETRAD NUMBERS 1st division:2nd division or ditype:tetratype	PERCENTAGE OF 2ND DIVISIONS OR TETRATYPES <sup>a</sup>	SOURCE
<i>Saccharomyces cerevisiae</i>	<i>ad, th</i>	(16+8):75	75.8 ( $p<0.05$ )	Lindgren, '49
<i>Podospora anserina</i>	<i>Hi(1)</i> , centromere	93:274	74.6	Hawthorne, unpublished
	<i>f</i> , centromere 26°	90:349	79.5	Rizet <i>et al.</i> , '49
	<i>f</i> , centromere 10°	36:273	88.3	Rizet <i>et al.</i> , '49
	<i>scx</i> , centromere 26°	25:969	97.5	Rizet <i>et al.</i> , '49
	<i>scx</i> , centromere 10°	13:1103	98.8	Rizet <i>et al.</i> , '49
	<i>i</i> , centromere 26°	92:332	78.3	Rizet <i>et al.</i> , '49
	<i>i</i> , centromere 10°	43:652	93.8	Rizet <i>et al.</i> , '49
	<i>i</i> , centromere . . .	52:210	80.2	Monnot, '53
<i>Centuria inaequalis</i>	<i>choline</i> (1905), centromere	7:47	87.0	Keitt <i>et al.</i> , '54
	<i>pantothenic</i> (2584), centromere	5:51	91.1	Keitt <i>et al.</i> , '54
	<i>purines</i> (2702), centromere	8:34	81.0 ( $p<0.05$ )	Keitt <i>et al.</i> , '54
	<i>purines</i> (3006), centromere	4:22	84.6 ( $p<0.05$ )	Keitt <i>et al.</i> , '54
	<i>histidine</i> (1261), centromere	14:62	81.6 ( $p<0.01$ )	Keitt <i>et al.</i> , '54
	<i>A, B</i>	21:86	80.4 ( $p=0.001$ )	Quintanilha, '33
<i>Coprinus finetarius</i>	<i>scx</i> , centromere	101:2024 (computed)	95.2	Hüttig, '31
<i>Ustilago hordei</i>	<i>scx</i> , centromere	(Numbers not stated)	98.7 ( $p=?$ )	Hüttig, '33
<i>Ustilago decipiens</i>	<i>yd, mt</i>	(0+1):18	94.7 ( $p<0.01$ )	Weaver, '52
<i>Chlamydomonas reinhardtii</i>	<i>cy, mt</i>	(7+4):87	88.8	Hartshorne, unpublished
	<i>cy, sc</i>	(4+4):86	91.5	Hartshorne, unpublished
<i>Sphaerocarpus donnellii</i>	<i>U, scx</i>	(1+3):43	91.5	Knapp, '36
	<i>G, scx</i>	(50+59):413	79.1	Knapp, '36
	<i>pd, scx</i>	29:103	78.0 ( $p<0.01$ )	Knapp, '37
<hr/>				
		TOTAL NUMBER OF DAUGHTERS ANALYZED	PERCENTAGE HOMOZYGOSIS $\times 4$	
<i>Drosophila melanogaster</i>	<i>cn</i> , centromere	8014	72.0	Beadle and Emerson, '35
(attached-X)	<i>cn</i> , centromere	516	73.6 (computed)	Bonnier and Nordenskiöld, '37
heterozygotes)	<i>sc</i> , centromere	8014	81.2	Beadle and Emerson, '35
	<i>sc</i> , centromere	40307	72.8	Rhoades, '31
	<i>y</i> , centromere	40307	75.6	Rhoades, '31
	<i>y</i> , centromere	1593	75.7	Morgan, '25
	<i>y</i> , centromere	22673	89.8	Bonnier and Nordenskiöld, '39

<sup>a</sup> Unless a  $p$  value is shown, the probability of exceeding 66.7% by an amount equal to or greater than observed is less than 0.001.

plish the reverse, and simulate chromatid interference where none existed. Unless such a mechanism exists, any departure from a random ratio of 2-, 3-, and 4-strand double exchanges provides valid evidence for the existence of chromatid interference. Consequently, investigations of chromatid interference in tetrads still remain meaningful, in spite of the possible existence of sister-strand crossing over.

Sister-strand crossing over could reverse the apparent sign of chromatid interference. But in order for sister exchanges by themselves to simulate chromatid interference between nonsister exchanges, a selective kind of chromatid interference would be required that affected nonsister-sister relations but not nonsister-nonsister relations. If sister-strand exchanges were numerous relative to nonsisters, as Schwartz ('53a) has suggested, one might expect chromatid interference to be most clearly demonstrable between very small adjacent intervals, and to be masked progressively in longer intervals. Data gathered by Huskins and Newcombe ('41) might be interpreted in this way.

A diagram (fig. 2) may be useful for summarizing the interrelations discussed. (1) Chiasma interference can be distinguished from chromatid interference in tetrads, but not in strands. (2) Tetrads, if adequately marked, are capable of providing a complete description of the positions and of the emerging types of all exchanges. (3) Whether the emerging types correspond to original types depends on whether sister-strand crossing over occurs. Its occurrence could affect both tetrads and strands by obscuring or reversing the sign of chromatid interference in tetrads, which would in turn alter coincidence in strands. (4) The diagram is incomplete in failing to show a direct relation between sister-strand crossing over, on one hand, and chiasma and chromatid interference, on the other. This omission is not necessarily justified by the demonstration that sister exchanges do not participate in chiasma interference on an equal basis with nonsisters, because a direct interrelation on some unequal basis has never been excluded.

It is apparent that the three inferred variables, chiasma interference, chromatid interference, and sister-strand crossing

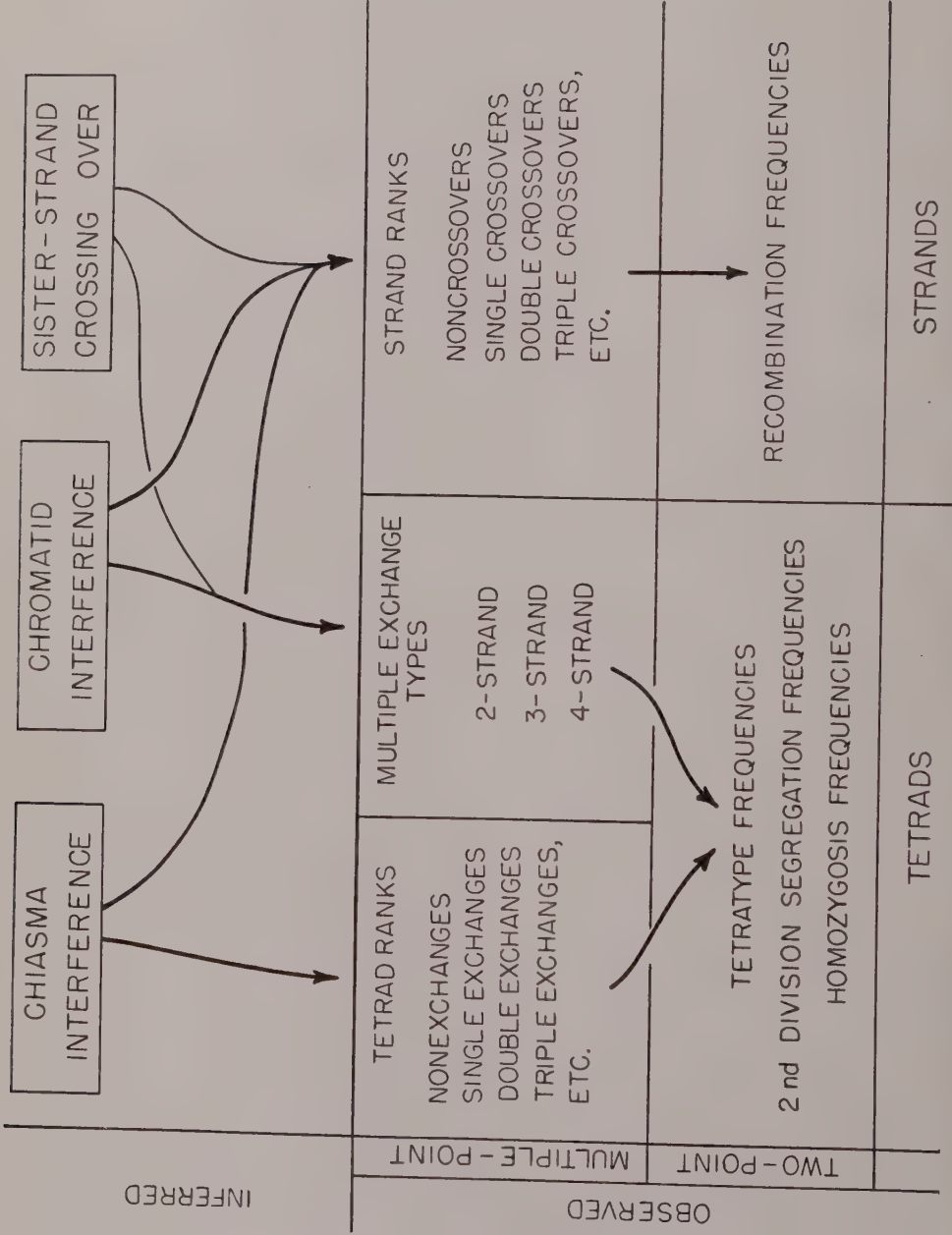


Fig. 2 Interrelations between inferred and observed variables in crossing over.



over, must be taken into consideration as fundamental features of any adequate theoretical model of crossing over (Weinstein, '36). This suggests a mode of attack that would depend largely on tetrads for its effectiveness, namely, to construct a basic model of crossing over, and to vary these fundamental features one at a time in such a way that their effects both on tetrads and on single strands could be predicted and compared with experimental data. I shall try to illustrate the feasibility of such an approach by using a simple model to predict tetra-type or second-division segregation frequencies for the one, and recombination frequencies for the other.

#### AN ILLUSTRATIVE MODEL

My model has the following basic features: (1) Crossing over occurs at the 4-strand stage. (2) Nonexchanges are not excluded for any interval. (3) Intervals contain no discontinuities such as centromeres or points of chiasma localization.

*Chiasma interference varied.* Let us begin by varying chiasma interference, assuming that chromatid interference does not exist, and that sister-strand crossing over either is absent or does not affect chiasma interference (fig. 3). The upper curves in figure 3 are for tetrads, the lower ones for single strands. (The solid curves for tetrads give the frequencies of tetra-types, or of second-division segregations, or of recessive homozygosis  $\times 4$ . Four-strand doubles and a fraction of multiple exchanges would produce nonparental di-type tetrads, shown by dashed lines to the same scale as tetra-types in figures 3, 5, and 7.)

Successive curves correspond to different amounts of chiasma interference. The straight lines describe the relations that would obtain with complete chiasma interference. The zero interference curves, at the other extreme, are based on a Poisson distribution of exchanges, and were obtained originally by Haldane ('19) for strands, and by Rizet and Engelmann ('49), and by Papazian ('51) (independently) for tetrads. The intermediate curves were obtained as follows.

It is assumed that when chiasma interference is varied for any interval between two marked loci, the probability of non-exchange tetrads remains unchanged, but that the proportions of tetrads with single, double, triple, and higher exchanges are

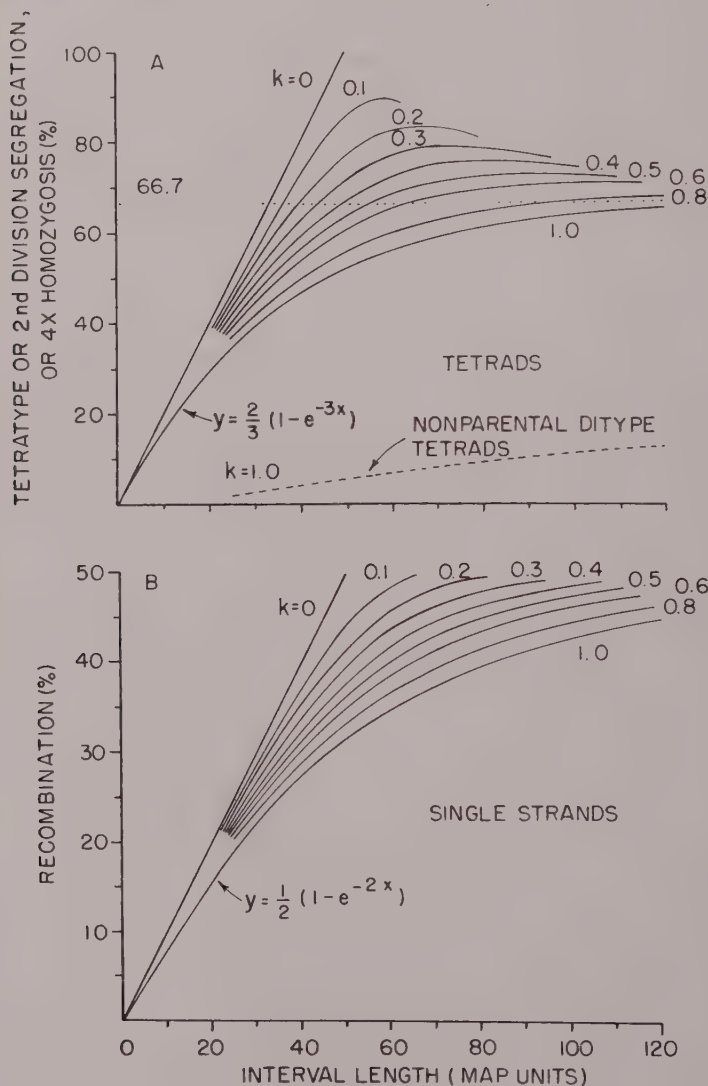


Fig. 3 Consequences of varying chiasma interference, in the absence of chromatid interference and sister-strand crossing over.

shifted. (Boost, '39 suggested this mode of attack, and called the length of an interval at zero interference the "*a priori*" map length.) The Poisson proportions that would obtain at zero interference were used as a reference, and new proportions for any specific amount of interference were determined by multiplying the successive terms of this Poisson expansion by a factor  $k^{r-1}$ , where  $k$  is a coefficient proportional to coincidence, and  $r$  represents the exchange multiplicity or rank of a tetrad. By varying  $k$  in the equations, chiasma interference is varied from being complete ( $k=0$ ) to being absent ( $k=1$ ). (For equations, consult Barratt *et al.*, '54.)

The tetrad curves characteristically pass through a maximum tetratype frequency (as indicated by Ludwig, '37). This is due to a predominance of single exchanges, and is consistent with data already presented in table 4.

Tetrad data from lower plants are still inadequate for curve fitting, even in *Neurospora*. But *Drosophila* data can be used for the present illustration. X-chromosome data for recombination frequencies and for homozygosis frequencies have been plotted in figure 4. They indicate that interference may correspond to the  $k=0.2$  or the  $k=0.3$  curves.

These attached-X data are unique in allowing interval lengths to be computed from component strands of the same individual that provided homozygosis frequencies. There are indications of a decline following the maximum in one set of the *D. melanogaster* data. A similar decline was shown for *D. virilis* by Demerec and Lebedeff ('34). The decline has been reported only once, that I know of, in lower plants. Knapp ('37) obtained 78% postreductions for a *Sphaerocarpus* interval 65 units long, falling to 65% when the distance increased to 93 units.

*Chromatid interference varied.* Let us now vary chromatid interference in the model, holding chiasma interference constant at the four levels shown in figure 4. The next two figures show the situation for two extremes of chromatid interference: completely positive (fig. 5), and completely negative (fig. 6). In the positive case, adjacent exchanges would be exclusively of the 4-strand type, giving rise to recombination frequencies greater than one-half. Tetratypes still reach a maximum

greater than two-thirds, even though all of them must come from single exchanges; they then decline rapidly toward one-half as a limiting value, instead of the normal two-thirds.

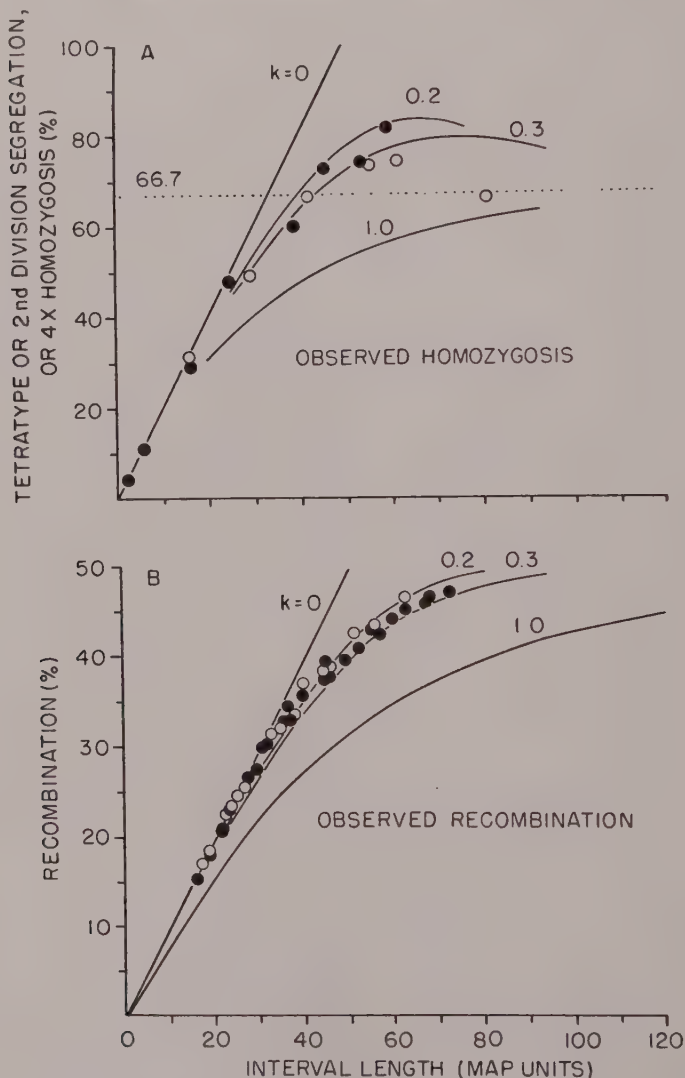


Fig. 4 Data from *Drosophila melanogaster* (X chromosome) compared with curves from figure 3. Interval lengths were determined individually for each set of data. A—solid circles, Beadle and Emerson, '35; open circles, Bonnier and Nordenskiöld, '37. B—open circles, Anderson and Rhoades, '31 compilation; solid circles, Morgan *et al.*, '37.

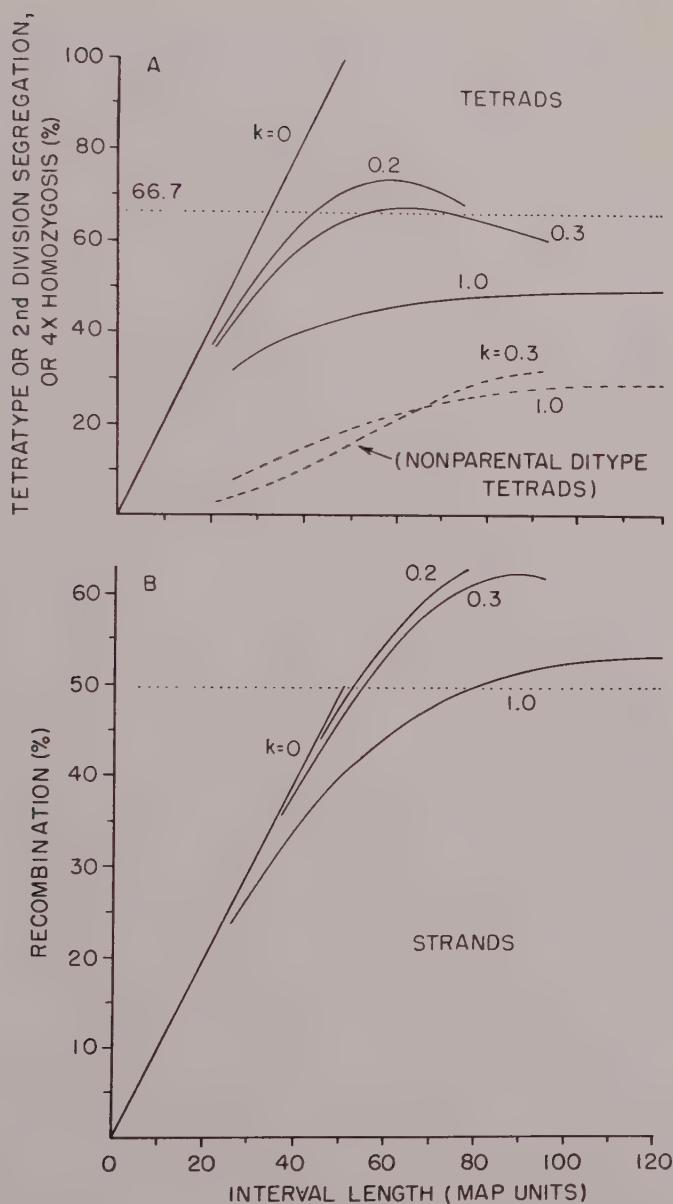


Fig. 5 Consequences of complete positive chromatid interference (adjacent exchanges exclusively of the 4-strand type) in the absence of sister-strand crossing over.



At the opposite extreme, all multiple exchanges are of the 2-strand type (fig. 6). Recombination values are depressed. Tetratype frequencies resemble those in figure 5, but non-parental ditype segregations are excluded. Both strand and tetrad curves are thus quite unrealistic.

*Sister-strand crossing over varied.* If sister-strand crossing over occurred independently of chiasma interference, and if it were frequent enough, all evidence of chromatid interference would vanish from figures 5 and 6, and the curves would become indistinguishable from those in figures 3 and 4.

One final set of curves in figure 7 shows what effect sister-strand exchanges would have if they participated in chiasma interference on an equal basis with nonsisters. Tetratypes could not exceed two-thirds, nor homozygosis frequencies one-sixth. This hypothesis was ruled out earlier by data transgressing these limits (table 4).

The model just used for illustration was originally developed for the practical purpose of estimating map distances with 2-point tetrad data from long intervals in *Neurospora* (Barratt *et al.*, '54). Because of its mathematical simplicity and its flexibility, it offers certain advantages over more elegant and specialized models. The feature to be stressed, however, could apply equally well to any 4-strand model (e.g., that of Carter and Robertson, '52). This feature is, that our model has been used to derive relations not only for single strands but also for tetrads. New possibilities for comparison and prediction are thus disclosed, that remain hidden so long as thinking is limited to single strands alone.

#### ORGANISMS FOR TETRAD ANALYSIS

It may be useful to conclude by describing the present status of some organisms that promise to be significant for tetrad analysis, and that may soon enable these theoretical relations to be tested. *Neurospora*, *Saccharomyces*, *Chlamydomonas*, and *Sphaerocarpus* are chosen as especially promising examples.

*Neurospora*. Knowledge of recombination in *Neurospora crassa* is by far the most extensive, thanks to the Lindegrens ('33-'42), to Houlahan, Beadle, and Calhoun ('49), and to many other investigators. More than eighty-five genes have

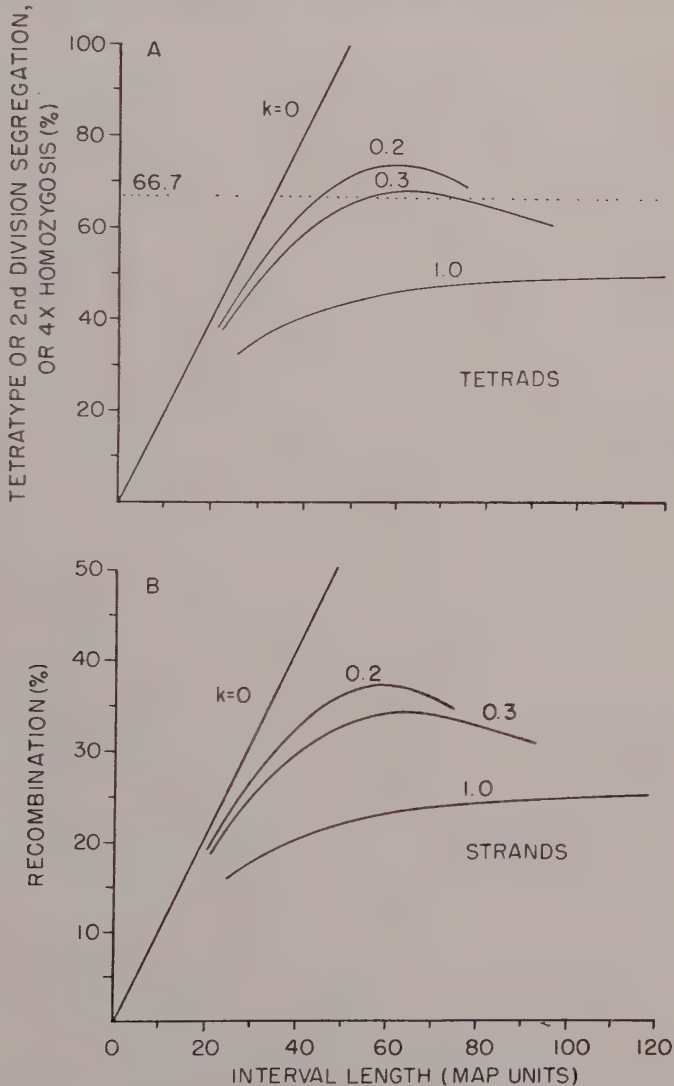


Fig. 6 Consequences of complete negative chromatid interference (adjacent exchanges exclusively of the 2-strand type) in the absence of sister-strand crossing over.

now been assigned to the seven linkage groups. Maps have been constructed from compiled tetrad data by Barratt *et al.* ('54).

Few multiple-point crosses have been made, so gene order is often uncertain. A majority of the markers are nutritional

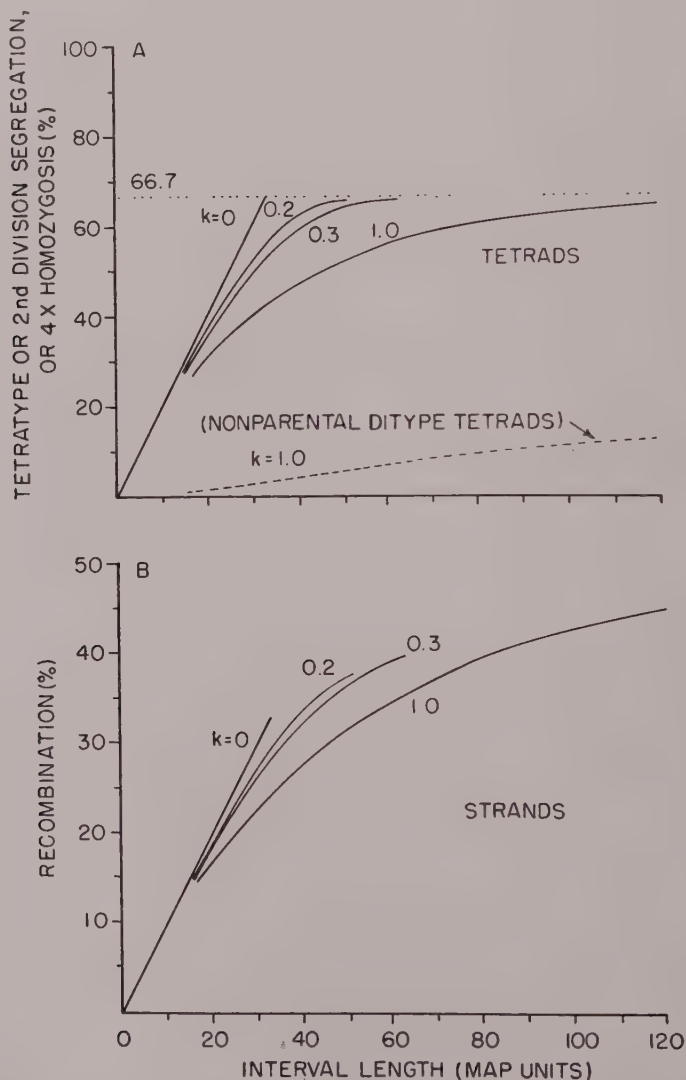


Fig. 7 Consequences of sister-strand crossing over that participated in chiasma interference on an equal basis with nonsister crossing over. Chromatid interference is assumed to be absent.

mutants, but there are a number of useful visible characters. Chromosome cytology is difficult but feasible (McClintock, '45; Singleton, '53), and translocations are already being used to assign linkage groups to specific chromosomes (Singleton, '48; St. Lawrence, '52).

*Saccharomyces*. The yeast *Saccharomyces cerevisiae* is less well known genetically than *Neurospora*, and is in several respects more difficult to analyze. Its use for recombination genetics has been retarded by the necessity of micromanipulation to isolate progeny, by a dearth of visible markers, and by the occurrence of irregular segregation ratios. Chromosome cytology is still too rudimentary to provide much help.

Despite these difficulties, substantial progress has been made. The first yeast map was published by Lindegren in 1949, 13 years after his first *Neurospora* map. Other genes have since been added (Lindegren and Lindegren, '51). Hawthorne ('53) has used linear yeast asci to map centromeres more accurately than was feasible with nonlinear ones (Whitehouse, '50; Lindegren, '49).

*Chlamydomonas*. Another organism that shows promise for recombination genetics is the unicellular alga, *Chlamydomonas*. Sonneborn ('51) has recently reviewed and evaluated the work of Moewus, so reference will be made here only to progress reported by other investigators.

Several species are being used, notably *C. reinhardi* (Smith and Regnery, '50; Weaver, '52; Hartshorne, '53; Nybom, '53; Sager and Granick, '53, '54; Sager, '54; Ebersold, '54; Eversole, unpublished); *C. moewusii* (Lewin, '52, '53; Nybom, '53); and *C. eugametos* (Nybom, '53; C. S. Gowans, unpublished). Technical advances in making crosses, in isolating and scoring segregants, and in detecting mutants suggest that *Chlamydomonas* may soon be handled with as great experimental facility as *Neurospora*. The cells of a tetrad can be separated manually, without recourse to micromanipulation, more rapidly than *Neurospora* ascospores can be isolated (Ebersold, '54). Preliminary chromosome studies have been made (Schaechter and DeLamater, '54).

A variety of morphological and physiological mutants are now available as markers. Among those that segregate as single genes are biochemical mutants with clear-cut requirements for arginine, *p*-aminobenzoic acid, nicotinic acid, thiamin, and acetate; mutants resistant to streptomycin, and to methionine sulfoxamine; mutants with impaired photosynthetic or heterotrophic capabilities; and pigment mutants. There are also a variety of morphological mutants involving such structures as chloroplast, eyespot, and flagella, as well as mutants recognizable by colony morphology.

Linkage has been shown in *C. moewusii* by Lewin ('53), and *C. reinhardi* by Ebersold ('54) who has mapped five genes (and the centromeres) in two linkage groups. The occurrence of tetratype segregations for the linked genes in both of these species proves that crossing over takes place at the 4-strand stage.

The occurrence of tetratype tetrads proves nothing regarding crossing over unless markers are known to be linked or unless the mode of centromere separation is known to be invariable, because tetratypes could result where there was no crossing over, or where crossing over was at the 2-strand stage, if centromeres sometimes divided equationally and sometimes reductionally at meiosis I or if centric activity was diffuse rather than localized.

I am indebted to the following persons for permission to use unpublished information concerning mutants and techniques: Mr. Russell A. Eversole (acetate, arginine, PABA, and nicotinic mutants in *C. reinhardi*), Mr. Charles S. Gowans (nicotinic and other mutants in *C. eugametos*), Dr. W. T. Ebersold (*C. reinhardi*), all three from Stanford University; and Dr. Ruth Sager, Rockefeller Institute for Medical Research (methionine sulfoxamine-resistant mutant in *C. reinhardi*).

*Sphaerocarpus*. The final organism chosen for comparison is a bryophyte — the liverwort *Sphaerocarpus donnellii*. This was first used for tetrad analysis by Allen ('24, '26) and later developed to a condition of great usefulness by Knapp ('36, '37) who mapped one linkage group containing seven gene loci and the centromere ('37).



The four meiotic products stay together in *Sphaerocarpus*, forming large, adherent spore tetrads that are easily separated from one another. Two male and two female plants result from each tetrad. X and Y chromosomes segregate regularly at the first meiotic division, and the morphologically recognizable sex difference thus marks the centromeres. Knowledge of chromosome cytology is well advanced (see, for example, Lorbeer, '41). In view of these characteristics it is surprising that *Sphaerocarpus* has not been used more widely.

These four organisms are by no means unique in possessing features favorable for tetrad analysis, and may well come to be rivalled by others such as *Venturia* (Boone, '51; Keitt, '52; Keitt and Boone, '54), *Glomerella* (Wheeler, '53; Chilton and Wheeler, '49), or *Podospora* (Rizet and Engelmann, '49; Monnot, '53).

We are now in a position to recognize the potential usefulness of tetrads. Materials are becoming available for realizing these potentialities, and as mapping improves, we may look increasingly to them for answers to some of the still unsettled questions regarding crossing over.

#### DISCUSSION

LINDEGREN: Since the  $P$  values (by  $\chi^2$ ) for regions I–IV and II–IV, based on Dr. Perkins' recalculated data, are 0.025 and  $<0.001$ , respectively, the evidence for chromatid interference is as positive on the hypothesis of misassortment of the centromere as on the hypothesis that the centromere segregates regularly at meiosis I.

PERKINS: It is true that two or three deviations that indicate chromatid interference still remain, but recomputation has decreased the deviations from 1:2:1 for all interval pairs that were originally deviant, except for II–IV.

J. LEDERBERG: I would agree we are sometimes too much wedded to the idea of regular prereduction of the centromeres, and that we ought not always to assume that no other possibilities exist. I wonder if either Dr. Perkins or Dr. Lindgren would comment on the situation in *Podospora anserina*, where

Rizet and Engelmann have shown that, in order to account for the segregation patterns in heterokaryotic ascospores, one would have to postulate a regular *postreduction* of the sex factor. To do this on the basis of regular *prereduction* of the centromere would require a single obligate crossover—in other words, absolute interference of chiasmata. But a possibly simpler interpretation would be, for the sex chromosome, *postreduction* of the centromere and close linkage of this with the mating-type factor. I do not know whether any factors on this chromosome have been found that show sufficient first-division segregation to rule out this explanation.

Some time ago, Dr. Lindegren showed me an unpublished manuscript which at least hinted that a paracentric inversion in *Neurospora* might interfere with pairing and lead to *postreduction* of the centromere.

PERKINS: I do not recall that Rizet or his collaborators have ever obtained markers properly placed in the compatibility group so as to accomplish this. But I believe that genes located in other linkage groups have provided critical evidence.

*Added note:* Data of Monnot ('53) on *Podospora* (Pleurance) exclude centromere *postreduction* as a general mechanism to account for the highly frequent occurrence of asci in which the binucleate ascospores are heterokaryotic for particular pairs of alleles, such as those governing compatibility. She reports crosses in which genes at two linked loci segregate so as to produce, in 69% of asci, spores that are homokaryotic with respect to one pair of alleles *S*, *s*, in contrast to producing only 20% of asci with spores that are homokaryotic for the other pair of alleles, *I*, *i*. At low temperatures the corresponding values are 98 and 6% respectively, which are in even more striking contrast.

Although centromere *postreduction* could readily account for segregations that resulted in as much as 100% of heterokaryosis for alleles that were presumably at proximal loci (say *I*, *i*, following Lederberg's suggestion), it would not allow more than 50% of segregations to be homokaryotic for genes located distally (say *S*, *s*), regardless of crossing over. The frequency of homokaryon-producing segregations in *Podospora* clearly exceeds 50% not only for the *s* alleles but also for genes at the *d*, *p*, and *b* loci, which attain 98, 94, and 61% homokaryotic segregations, respectively (Rizet and Engelmann, '49).

The fact that some genes segregate so as to be homokaryotic in practically all asci, while other genes are almost always heterokaryotic in the same asci, cannot easily be reconciled with the second mechanism suggested by Lederberg, especially when the contrasted genes are linked as in Monnot's experiments. But these results are compatible with the hypothesis adopted by Rizet *et al.* ('49) that centromeres are prereduced normally, and that heterokaryotic ascospores result from the occurrence of single crossing over in the interval between centromere and gene locus.

MICKEY: I should like to recall that there is a classic example of equational first division of the sex chromosome in the squash bug, *Anasa tristis*. In the male of this insect, which is the X-O type, the X chromosome regularly divides equally in the first meiotic division, then goes to one pole but not to the other in the second division. Although the chromosomes of hemipterous insects are considered to have diffuse spindle attachments rather than typical localized centromeres, it appears that this case is still pertinent to a discussion of segregation and crossing over.

SAGER: I should like to point out the usefulness of tetrad analysis in the detection of cytoplasmic inheritance in organisms in which both parents contribute their total cell contents to the zygote.

One type of nonchromosomal inheritance, detected in yeast and in *Chlamydomonas* by the occurrence of 4:0 segregation, has been attributed to a nonchromosomal unit present in many replicates and distributed randomly at meiosis. In these instances the ruling out of known chromosomal mechanisms has been greatly simplified by the recovery of all four products of meiosis.

Work with chloroplast mutants of *Chlamydomonas* has posed the problem of how to detect another type of nonchromosomal inheritance, if it occurs; namely, the direct inheritance of a unit cytoplasmic body in which crosses between two contrasting forms would presumably result in regular 2:2 segregations. Such factors could be distinguished from Mendelian ones by the absence of linkage with other markers, but this requires an exceedingly well-marked genome. Preliminary

screening can be provided by tetrad analysis, on the assumption that the regular distribution of a cytoplasmic unit would require an oriented segregation at meiosis, most likely all first- or all second-division segregation, and not the intermediate frequencies of second-division segregation exhibited by most chromosomal loci, owing to centromere distance and crossing over. Since with tetrad analysis one can compute the frequency of second-division segregation for each of three or more unlinked markers in multifactorial crosses, a means is available to detect instances of complete linkage with an independence of the centromere, and thus to sort out possible instances of nonchromosomal inheritance for further study.

PAPAZIAN: I think it best to keep an open mind as to the most useful way of measuring chromatid interference. To consider it as similar to chiasma interference where one break inhibits or promotes the probability of another break close by may be wrong; it may be that the proportion of symmetrical (2- and 4-strand) doubles to asymmetrical (3-strand) doubles is a more appropriate measure. Other patterns are also possible. The effect of temperature on interference is certainly of great importance. In Rizet's experiments with *Podospora*, it was proposed that a single crossover invariably occurred between the centromere and the mating-type locus. A locus was known in this interval and the frequency of second-division segregation of this locus varied greatly with temperature. This was taken to mean that the average position of the single invariable crossover varied with temperature. The regular second-division segregation of the mating-type locus could well be due to second-division segregation of the centromere, as Dr. Lederberg suggested, but supplementary hypotheses would have to be made to account for the behavior of the locus in the interval between centromere and mating-type locus. Moewus' account of the occurrence of crossing over at the 2-strand stage at temperatures below 5°C. can be regarded as an upset of the normal time of division of centromere relative to chromosome; or as a case of constant 4-strand doubles. The



effect of temperature, and especially low temperature, on interference needs further investigation.

DeLAMATER: Since Dr. Perkins has emphasized the possible use of various microorganisms as cytogenetic tools, it is perhaps proper that a few comments should be made about the cytology of *Chlamydomonas* and *Schizosaccharomyces octosporus*. This work has been done in collaboration with Drs. Moselio Schaechter and Abraham Widra of my laboratories. During the course of the last 2 years we have studied both the mitotic and the meiotic processes in representative members of these two groups.

In *Chlamydomonas* at the metaphase stage, it is possible to count the chromosomes with great clarity, as have Pocock and Cave in other volvocales. The chromosome number in *C. moewusii* is 36, which is considerably over the expected number if we accept the linkage groups as defined by Moewus. The meiotic phenomena in this organism are very peculiar and are complicated by the extremely small size of the chromosomes. There appears to be an aggregation of two or more non-homologous chromosomes during the meiotic prophase, so that ten discrete bodies appear in a ring configuration at the time of diakinesis. This number corresponds with the number of linkage groups described by Moewus. Further study will be necessary before this picture is completely understood.

In *Schizosaccharomyces* the haploid chromosome number appears to be 4, and the meiotic configurations, in contrast to those found in *Chlamydomonas*, appear to follow the classic patterns more closely.

It appears to me that the major limitation of these organisms as cytogenetic tools is the minute size of the chromosomes which they possess.

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# GENETIC MEASURES OF CENTROMERE ACTIVITY IN *DROSOPHILA MELANOGASTER*<sup>1</sup>

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## TWO FIGURES

The great diversity of chromosome types available in *Drosophila melanogaster*, the precision with which these can be defined by the use of the salivary gland chromosome technique, the relative abundance of mutant genes, and the remarkable extent to which the chromosomes in this species may be manipulated genetically, all provide an unusual opportunity for the study of the behavior of chromosomes as organized units possessing characteristics not immediately attributable to their specific gene content. Moreover, the vast amount of genetic data accumulated over the years with this species provides a definite set of expectations in most experiments; deviations from these may furnish a clue to some previously unrecognized phenomenon.

One exceptionally fruitful approach to problems of chromosome behavior at the meiotic divisions involves the deliberate attempt to study chromosomes of a complex nature. In such situations, the consequences of exchange and disjunction are theoretically predictable, but there often appear discrepancies between the expected and observed results. Examination of the assumptions on which the predications are based may show one or more of them to be suspect. The validity of these assumptions may then be tested in less complex situations.

The experiments to be discussed here represent a genetic attack on the properties of the centromere. It may not be im-

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mediately obvious that this is feasible in *Drosophila*, for such a study requires some sort of device for measuring centromere activity genetically. Three such devices are to be considered here: (1) where two centromeres, with their adjacent heterochromatic regions, have different origins and are on opposite ends of a double first anaphase bridge; (2) where a chromosome does not pair with a homolog and is lost with some frequency during the meiotic divisions; and (3) where sister centromeres, at opposite ends of a second anaphase bridge, are tied together by two chromatids of the same or of different lengths.

#### COMPARISON PROVIDED BY DOUBLE FIRST ANAPHASE BRIDGES

The interpretations of Sturtevant and Beadle ('36) concerning the behavior of inversion heterozygotes provide the framework within which certain results to be discussed must be initially considered. From simple inversion heterozygotes, the only recovered crossover products come from double or, less commonly, higher-rank exchange. The 2- and 3-strand doubles produce recognizable crossovers, while the 4-strand doubles result in double first anaphase bridges. Because a predictable ratio of three observed crossovers to two patroclinous males is fulfilled, it was concluded that these bridges give rise to nullo-X eggs which, following fertilization by an X-bearing sperm, produce patroclinous males.

Examination of data from females heterozygous for a ring chromosome showed that the observed proportion of patroclinous males was much lower than the expectation based on the same kind of analysis as that just indicated (Novitski, '52). A line drawing of the bridges arising in the two types of females reveals that they differ in one important respect: in the inversion heterozygote, the dicentric chromosomes formed by exchange are characterized by subterminal centromeres, whereas those formed in ring heterozygotes, although having one subterminal centromere, have one centromere ultimately derived from an attached X, which has appended to it a free arm consisting of an entire X chromosome. It was

considered possible that the asymmetry of the bridges and the reduction in patroclinous males were causally related. Because the ring chromosome-normal chromosome combination is already moderately complex and conclusions based on results from it alone may be justifiably suspect, this idea was tested by studying the results from an inversion heterozygote which could produce bridges mimicking those described. This was accomplished by using as the chromosome in normal sequence a two-armed X chromosome derived from attached X's through crossing over with the Y. Such an exchange produces reciprocal products each carrying an X chromosome with a Y chromosome arm attached as an extra arm. One recombinant carries the centromere of the Y chromosome and the other the centromere of the attached X. Since attached X's are ordinarily formed through successive exchanges between the X and Y chromosomes, it seems quite likely that attached-X centromeres are, in some cases, at least, ultimately derived from a Y chromosome.

The proportion of patroclinous males recovered from females heterozygous for an inversion and a chromosome in normal sequence with the long arm of the Y chromosome appended was found to be approximately half of the expectation based on studies of simple inversion heterozygotes. Since the four products of meiosis in the egg of *Drosophila* arrange themselves in a linear fashion with one of the terminal nuclei of this linear array becoming the functional egg nucleus, it seemed reasonable that the orientation of the asymmetrical bridges with respect to the egg nucleus might account for the observed discrepancy. These bridges may orient in two ways with respect to the egg nucleus, and if one of the orientations should fail to give rise to patroclinous males the new expectation would agree with the observation. Reasoning of this type leads to a tug of war analogy in which the asymmetric bridge is presumed to bear centromeres of unequal strengths, such that the entire bridge configuration is dragged to one pole by the stronger centromere. Such a phenomenon should lead to the delivery, in one-half of the cases, of an

intact dicentric (or some product of a dicentric) to the functional egg nucleus which would later kill the zygote. A logical sequel to this experiment is the combination in which both the inverted and the normal sequences carry a second arm, thus reestablishing the symmetry. The expectation here is that either the bridges would once again be stalled on the spindle or that the bridges would fragment. The first case would lead to the same frequency of nullo-X eggs as in simple inversion heterozygotes, while the second would lead to the delivery to the functional egg nucleus of fragmented chromosomes reducing the frequency of the patroclinous male class to almost zero. The latter proved experimentally to be the case, and hence the results are consistent with the supposition that the centromere with a Y arm appended is "stronger" than the subterminal X chromosome centromere.

It is questionable whether the observed genetic differences among the various chromosome types are attributable to essentially different centromeres, as such, or to the additional chromosome material present as an extra arm. The difficulty in making a distinction between these two possibilities experimentally arises from the lack of specific information about the exact nature of the chromosomes used. First, there is some ambiguity about the origin of the centromere of an attached-X chromosome due to the fact that the attached X's are usually formed in a series of successive crossovers between the X and the Y chromosomes, and depending on the exact position of the exchanges, may have an X centromere or a Y centromere, and, furthermore, there must be heterochromatic segments of indeterminant lengths derived from the Y chromosome at the base of an attached X. Finally, the detachment comes about as a consequence of an exchange between the attached-X and Y chromosomes and, although by simple genetic tests it is possible to determine which arm of the Y chromosome is carried by the detachment, it becomes difficult (if not impossible) to specify the origin and nature of the other heterochromatic segments carried by the detachment. Furthermore, the lack of good genetic markers in these regions and an



apparent nonspecificity of exchange between the heterochromatic regions of the X and Y chromosomes of *D. melanogaster* (Lindsley, '54) may make the interpretation of any result difficult.

#### THE MEIOTIC LOSS OF UNPAIRED CHROMOSOMES

The second method which may reveal something about centromere activity comes from an apparently unrelated study of the behavior of unpaired chromosomes, i.e., univalents, in *Drosophila* (Sandler and Braver, '54). It has been shown, entirely by genetic methods, that such unpaired chromosomes may be lost during the meiotic divisions, and in some instances with a frequency which exceeds 50%. This effect is probably analogous to the loss of univalent chromosomes in plants which has been known for a long time.

From the point of view of this discussion, however, the most significant observation was that the attached-X chromosome without a homolog, i.e., the Y chromosome, showed no detectable frequency of loss, and the attached-XY chromosome (an X chromosome with the long arm of the Y chromosome attached to the centromere and the short arm attached to the tip) shows a very much lower frequency of meiotic loss than an unpaired Y chromosome. The conclusion from this was that a requirement for the regular passage of chromosomes to daughter nuclei during meiosis is paired heterochromatic regions adjacent to the centromere, irrespective of whether these regions are on separate chromosomes or on the same unpaired chromosome.

#### RESULTS FROM AN ANALYSIS OF THE TANDEM RING X CHROMOSOME

The study of the tandem ring chromosome has led to some interesting conclusions about (1) the behavior of second anaphase bridges, (2) the possibility of the formation of interlocked complexes in such a chromosome after crossing over and, incidentally, (3) the results expected from sister-strand



exchange in ordinary ring heterozygotes. Each of these will be considered in detail.

*The structure and meiotic behavior of the tandem ring chromosome.* This chromosome consists of two X chromosomes, joined end to end to form a continuous ring, with the two components arranged in tandem such that they synapse spirally (fig. 1). Such a chromosome should be able to cross over with itself; diagrams indicate that various types of exchanges should give rise to single ring chromosomes, double

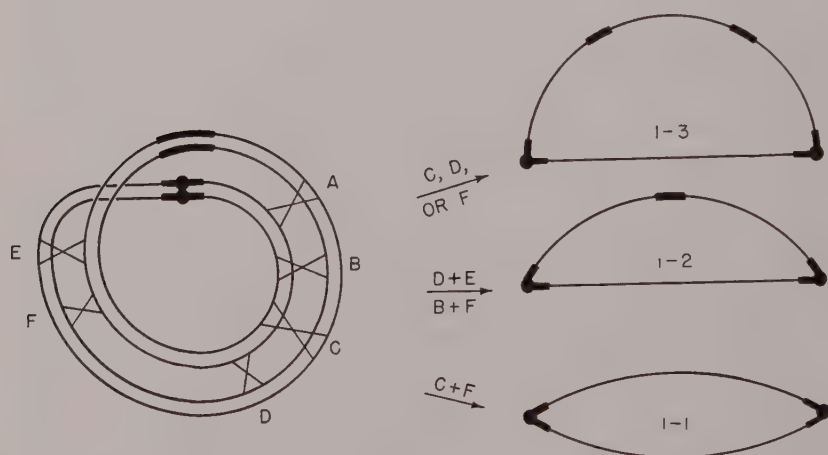


Fig. 1 Synapsis of the two components of the tandem ring, showing the essentially different kinds of exchanges that give rise to the three different sorts of second anaphase bridges.

ring chromosomes, triple ring chromosomes, acentric rings, and a variety of dicentrics (Novitski, '54).

To understand the results obtained from a study of this chromosome, it should be kept in mind that this is a complicated kind of attached-X chromosome. At the first meiotic division it disjoins from its homolog, usually a Y chromosome. Since one of the terminal meiotic products is destined to become the egg nucleus, the orientation of the two homologs at metaphase I will determine whether the compound X chromosome or the Y chromosome will be included in the

egg nucleus (as in top line of fig. 2). This orientation has never been shown to be anything but random, and consequently one expects half the eggs to be Y-bearing and nullo-X and the other half to carry the compound X chromosome or some crossover product from it. Thus the number of patroclinous males should serve as a measure of the number of eggs receiving the compound or one of its derivatives.

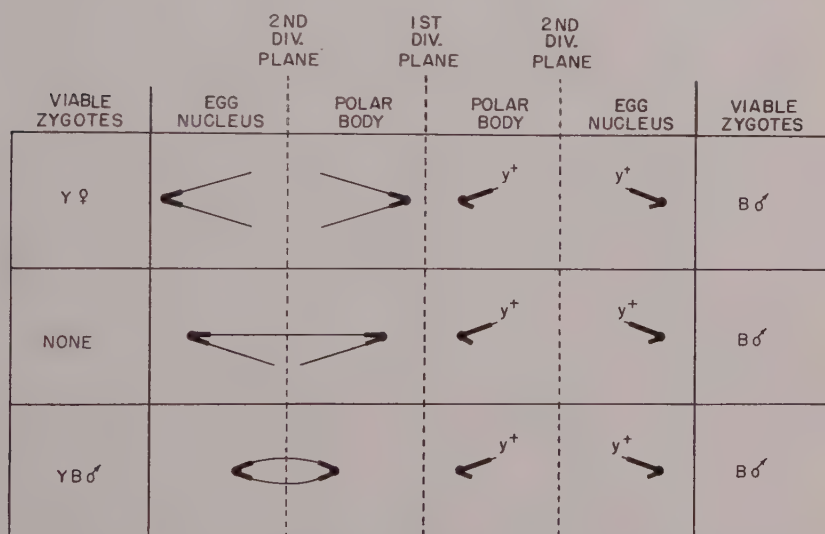


Fig. 2 Schematic diagram of the segregation of a compound (in this case, the tandem metacentric) from a homolog marked with the normal allele of yellow. The phenotypes of the viable zygotes, when the male parent is yB, are given in the outer margins. The first row shows the linear array of meiotic products when there is no second anaphase bridge, the second when there is a simple bridge that fragments to give rise to a lethal zygote and the third, when there is a 1-1 bridge from a double exchange stalled on the spindle and excluded from the egg nucleus.

The initial experiments with this chromosome gave a ratio of patroclinous males to X-bearing progeny in excess of that expected after taking into account the lethal classes from dicentrics (Novitski, '54). Part of this excess may come from a lowered viability of the tandem ring, but a question arises as to whether there is an additional source of nullo-X eggs

from this compound. If the consequences of exchange in this compound are examined in detail, it can be shown that some of the commonly expected products are second anaphase bridges. These are, however, different from the usual type of anaphase bridge (which has been shown to give rise to lethal zygotes in other types of experiments) but are unique in that they possess two chromatids tying sister centromeres together. These bridges, moreover, are not identical for all combinations of exchanges. There are, in fact, three distinct common types (fig. 1). After a single exchange, the two chromatids tying the centromeres together are grossly unequal in length, one being as long as a single X chromosome and the other as long as three X chromosomes. Of the eight essentially different kinds of double exchanges, two produce dicentrics which have a link two X chromosomes long and a link one X chromosome long, and one of the eight double exchanges produces a dicentric with two chromatids of the same length, each being one X chromosome long. Symbolically, a distinction can be made among these three kinds of double second anaphase bridges by taking the length of one X chromosome as a unit and representing the three classes as 1-3, 1-2, and 1-1 bridges, respectively.

*Products from second anaphase bridges.* The possibility that the second anaphase bridges just described may be lost on the second division spindle and thereby give rise to a new source of nullo-X eggs may be easily tested. If the homolog of the compound X is genetically marked, then the nullo-X eggs which arise in the usual way must carry this marked chromosome. Any arising by loss for some reason of the compound chromosome would not carry the homolog (lower line of figure 2). The results from an experiment designed to yield this type of information are given in lines 1 and 2 of table 1 where it can be seen that about 10% of the patroclinous males arise from the failure of the compound ring, or one of its derivatives, to be included in the egg.

To determine whether these double chromatid dicentrics are ever eliminated from the functional egg, the marked homo-

log type of experiment was made with the tandem metacentric (or tandem attached X), which had previously been investigated in detail (Novitski, '51). In a compound of this sort, second anaphase bridges are produced by crossing over, all the bridges coming from single- and 3-strand double exchanges are the same, having a single strand between the two centromeres. However, half the 4-strand double exchanges give rise to bridges similar to the class 1-1 bridges produced by the tandem ring. Although the argument for the lethality, in general, of second anaphase bridges from this compound

TABLE 1

*Progeny produced from matings of females carrying compound X chromosomes homozygous for yellow (except for the Hw f tandem metacentric), and a Y chromosome fragment, FR2, with the normal allele of yellow, to males carrying the X-Y chromosome marked with yellow and Bar*

TYPE OF COMPOUND	REGULAR CLASSES		GENERATED RINGS		EXCEPTIONAL CLASSES		PERCENT-AGE EXCEPTIONAL $\frac{y B \sigma}{y B \sigma^+ B \sigma}$
	$B \sigma \sigma$	$y \varnothing \varnothing$	Non- $B \sigma \sigma$	$B/+ \varnothing \varnothing$	$y B \sigma \sigma$	Non- $y \varnothing \varnothing$	
Tandem ring	2530	245	913	766	254	3	10.4
Tandem ring	8166	959	2842	2957	700	7	8.6
Tandem meta-centric (Hw f)	2763	681	1205	1049	136	..	4.9
TM, dl-49/+	5823	4611	813	429	30	7	0.5
TM, +/+	1138	246	187	92	50	2	4.4

is very strong (Sturtevant and Beadle, '36; Novitski, '51), the possibility that this one specific type of bridge did not give rise to lethal zygotes had not previously been considered. Since it was known that crossing over takes place at a normal rate in this type of compound, it could be predicted that, as a minimum estimate, there should be 3% of such bridges, and, if the marked homolog method was used to distinguish between ordinary patroclinous males and those from nullo-X eggs arising after such bridge formation, at least 3% of the patroclinous males should be of the new type. This is a minimum estimate because the tandem metacentric had

been marked at only two loci, and therefore homozygosis, which gives the measure of double crossing over, could not be measured completely, whereas detection of the bridges would not depend on heterozygous loci in the compound.

The results from a cross designed to measure the frequency of patroclinous males produced from nullo-X-nullo-Y eggs from a tandem metacentric compound X chromosome are given on line 3 of table 1. There were, in this experiment, 4.9% of such exceptional individuals. Now it is reasonable to question this result, for this value might simply represent nullo-X-nullo-Y eggs from some variable proportion of the two types of bridges produced by this compound; that is, those with one and those with two chromatids connecting the centromeres. Furthermore, it was not possible to measure primary nondisjunction in this run. To settle these questions a new tandem metacentric compound was constructed which was heterozygous for the dl-49 inversion and homozygous for the recessive *y*, making nondisjunction detectable. It is known that the effect of the heterozygous dl-49 inversion in this compound is to reduce the frequency of single exchanges drastically in the region of the inversion and distal to it, but to allow single exchanges to occur with about normal frequency from the centromere region to the proximal breakpoint of the inversion (Novitski and Braver, '54). As a consequence, single exchanges in such a heterozygote may occur with a high frequency whereas doubles are virtually eliminated. If the exceptional patroclinous males come predominantly from eggs with these double bridges, then there should be a drastically reduced frequency of such males. If, on the other hand, both types of bridges produce such males, the frequency from this compound should not be reduced drastically because single exchanges, which produce bridges, still occur with a high frequency. From table 1, line 4, it can be seen that this compound gives only 0.2% exceptional males. Once again it might be imagined that this result is spurious; that this new tandem metacentric is simply behaving differently from the previous types. This can be tested by removing the dl-49 in-



version (by isolating a rare double exchange type); this will allow crossing over to occur freely in the compound. Such a line was isolated and tested; it gave a frequency of exceptional patroclinous males of 4.2% (line 5, table 1).

It may now be considered demonstrated that the lethality of second anaphase bridges does not extend to the bridge of class 1-1. From this observation, the most reasonable interpretation is that these equal-strand, one-chromosome-long double bridges are lagging on the second division spindle and getting lost, whereas single chromatid bridges are fragmenting at the second division; the inclusion of the fragment into the functional egg nucleus eventually kills the zygote. Prior to this observation there appeared to be no reason for believing that the simple dicentrics might not persist as dicentrics and, as such, cause lethality. With this hypothesis of lethality caused by fragmentation in mind, let us reexamine the three types of dicentrics produced by the tandem ring. Although all three have two strands connecting the centromeres, only one has strands of equal length; in the absence of chromatid interference, the other two classes should be more frequent than this latter class and, in particular, class 1-3 should be most common, since it comes from half of the single exchanges. From the relatively low percentage of nullo-X-nullo-Y eggs produced, it can be argued that those dicentrics with two unequally long chromatids are producing lethality; i.e., there is breakage of the two strands, presumably one at a time, a crude analogy being the breakage of two ropes in succession, the shorter being broken first.

*Interlocked complexes.* If crossing over is visualized as occurring in a three dimensional system, it might be expected that the various kinds of rings would be so intertwined after crossing over as to give a very high frequency of loss; in fact, it was anticipated that the stock of the tandem ring might eliminate itself by crossing over. It proved, however, to give results not far from the expectations based on a line drawing. Quite clearly, the chromosome either does not regularly get involved in complex interlocks in the first place, or,

if it does, it possesses some special faculty for getting out of this difficulty.

There is a way of making a crude estimate of the maximum allowable loss by interlocking. From single and double exchanges the simplest expectations are 1 single ring: 1 double ring: 2 lethal zygotes (the last class arising from triple rings as well as dicentrics). The data in table 1 show that more than 25% of the products of meiosis are single rings, and that the ratio of single rings to double rings is not 1:1. (In the experimental results given on lines 2 and 4, the male parent had the X-Y chromosome with an additional Y chromosome as a homolog. The other experiments involved the use of a univalent X-Y chromosome, which shows some meiotic loss, usually about 16%, and the more valid data on the tandem ring are therefore those given in line 2. These comparisons then are  $(2957 + 2842)/2 \times 8166 = 35.5\%$  and  $(2957 + 2842)/2 \times 959 \neq 1/1$ .)

Although the deficiency of double rings suggests a drastically lowered viability of this compound, the two relations mentioned, particularly the first, suggest that nonrandom disjunction, shown to exist where single rings are produced by the tandem metacentric, is also operating here. This, in itself, may be considered evidence that there is little interlocking of rings after crossing over, because in those cases investigated in *Drosophila* where nonrandom disjunction has been observed (tandem metacentric and inverted chromosomes, Novitski, '51; translocation heterozygotes, S. Zimmering, unpublished), it seems to depend on some structural difference between homologs. Any structural differences between the two chromatids making up a dyad should be obscured if they were wound around each other.

In any case, if it is assumed that the coefficient of non-randomness in this case is 1, that is, that single rings produced by crossing over are always included in the functional egg nucleus to the exclusion of the complementary product, and if it is further assumed that there is no loss from inviability of rings, and finally that all tetrads carry at least one

exchange, it can be readily shown from the data given in line 2 of table 1 that the maximum allowable loss from interlocking is about 20%. Since relaxing each of these assumptions reduces the amount of such loss, and, in fact, the magnitude of each of these effects must be less than assumed here, the actual loss by interlocking must be considerably less than 20%, if any occurs at all.

We have now considered the arguments against the loss of any more than about 10% of rings by interlocking, and subsequent stalling at second anaphase, and those against the loss of more than 20% of the rings by interlocking, followed by lethality to the zygote. There remains but one other possibility if interlocking is common, and this is simply that the interlocked rings break at second anaphase with the free ends subsequently rejoining to reconstitute normal rings. This is obviously impossible to eliminate decisively, yet there are two arguments against it. One, already mentioned, is that the structural distinction between the single ring chromosome and its complement must be present prior to the second division in order to give nonrandomness; how such a distinction could be evident in an interlocked complex is by no means obvious. In the second place, roughly half the single rings produced by crossing over originate simultaneously with acentric rings. Breakage of one of the centric interlocked rings would leave the other with the acentric still interlocked. The presence of an acentric ring interlocked around a centric ring might cause difficulty during the early cleavage divisions; if such were the case, the occasional loss of an X chromosome during cleavage would give rise to gynandromorphs. These do not seem to occur with any unusual frequency in these experiments.

*Sister-strand crossing over.* Another aspect of this production of the nullo-X eggs, and, consequently, patroclinous males in those cases where the double bridge does not break is of some interest. These bridges are like those which would arise from a single sister-strand crossover (or an odd number of such) in a ring chromosome. This similarity is more than

generic: both the tandem ring and the tandem metacentric compound have been derived ultimately from single ring chromosomes and the double-strand second anaphase bridges from these compounds must be structurally very similar to the bridges which would be produced by sister-strand crossing over in those single rings. In fact, the centromere regions and adjacent heterochromatin should be identical in the two cases.

The frequency of patroclinous males from females heterozygous for a ring chromosome is of interest once again, since, if sister-strand crossing over were occurring in the ring, one would expect an increment of patroclinous males from this source. It will be recalled, however, that the ordinary ring heterozygote produces a deficiency of patroclinous males; this observation had led to the experiments on symmetrical and asymmetrical first anaphase bridges. The ring-rod heterozygote experiment may be modified slightly in a way suggested by L. V. Morgan ('33), who first checked for an excessive loss of the  $X^{c1}$  ring by sister-strand crossing over. If an inversion is inserted into one of the two homologs, the products of normal crossing over will be less frequent, providing the better opportunity to observe any effects of sister exchange, if it should occur. The inversion used here was  $In(1)AB$ , and the parental males were marked so that patroclinous males could be detected. The maternal X chromosomes recovered in the  $F_1$  were as follows: 12,487 non-crossover rod chromosomes, 11,499 noncrossover ring chromosomes, 47 double crossover rods, 19 double crossover rings, and 396 nullo-X (patroclinous males). Thus it can be seen that the maximum loss of ring chromosomes by sister-strand crossing over is about 3.2% ( $396/12,487$ ), and this figure must include patroclinous males arising by primary nondisjunction, and also from the usual types of anaphase bridges.

This argument against the occurrence of sister-strand crossing over in the single ring of *Drosophila* does not, however, extend to the results from the tandem ring chromosome. The modification of Belling's theory of crossing over suggested by Weinstein ('36), by Lindegren and Lindegren ('37),



and by Schwartz ('53b), according to which only two of the four chromatids take part in exchange initially with sister exchange later randomizing the regions of exchange among all four chromatids, leads to expectations somewhat different from those given by the standard analysis followed here, specifically, a decreased recovery of double rings and an excess of nullo-X-nullo-Y eggs. The data from the tandem ring do, in fact show a deficiency of double rings and an excess of exceptional patroclinous males. Unfortunately, since these discrepancies may also be attributed to other factors (the level of crossing over is indeterminable in this case because of the absence of heterozygous markers, the viability of the tandem ring is open to suspicion, nonrandom disjunction appears to be occurring and altering the simple expectations on any hypothesis) an unequivocal answer must await the analysis of additional compound rings.

#### CONCLUSIONS

The reality of the spindle body and its role in chromosome movement during mitosis of most plant and animal cells seems established beyond any reasonable doubt (Schrader, '53; Mazia and Dan, '52). That the chromosomes themselves may make a contribution to the chromosomal fiber, suggested as early as the latter part of the last century, and elaborated in detail by Belar, is supported by the birefringence studies of Schmitt, and of Swann. Detailed reviews of the evidences are found in the works of Schrader ('53) and Swann ('52). Recently, Rhoades ('52) has described the formation in certain strains of maize of supernumerary chromosomal fibers at localized regions (neocentromeres) on the chromosome. This strongly suggests a contribution of the chromosomes themselves to the fibers; the genetic data from experiments on the behavior of anaphase bridges and on chromosome loss are consistent with this possibility. Indeed, they appear to be difficult to explain on any other basis.

It has been shown genetically that dicentrics may differ from one another at first anaphase of meiosis and these dif-



ferences depend on the origin of the centromere and adjacent heterochromatic regions. That these results may be ascribed to varying levels of "strength" of the centromere, simple and convenient for the purpose of description, implies a fundamental difference in the nature of centromere regions of various origins, but, as was pointed out in detail in the text, it is not possible from the data now available to make a distinction between the contribution of the centromere itself and that of the adjacent heterochromatin, since all centromeres of the so-called strong variety are ultimately derived from the Y chromosome along with some indeterminate amount of Y chromosome material.

The possibility that such differences might originate in some segment of the chromosome other than the centromere is supported by the mentioned work of Rhoades on neocentromeres and is suggested also by the observations on meiotic loss of unpaired chromosomes in *Drosophila* by Sandler and Braver ('54), who found that, in general, univalents are lost with an appreciable frequency, as they are in plants, but that the attached-X chromosome behaves normally both with and without a homolog. Therefore, it can be surmised that the composition of the chromosome itself, independent of the presence of a homolog, must play some part in determining normal movement during the meiotic divisions, and that the two arms of this chromosome make the same contribution to fiber formation that they would make if they were present separately as two homologous chromosomes.

The production of lethal zygotes by certain kinds of first and second anaphase bridges, and not by others, can be attributed to the resistance of the chromatid to fracture when subjected to forces of varying degrees. The recovery of the theoretically expected number of nullo-X eggs from inversion heterozygotes involving X chromosomes, the halving of this number when one of the chromosomes in the heterozygote is replaced by a derivative of an attached-X chromosome, and the virtual elimination of this class when both chromosomes are of this latter type form a consistent picture based

on the crude mechanical analogy of a tug of war. This is reinforced by the data from the compound X chromosomes on second anaphase bridges from which it seems clear that two sister centromeres opposing each other can fracture either a single chromatid, or two if they are unequal in length, but not two identical chromatids simultaneously.

The evidence that the tandem compound ring chromosome does not regularly become involved in complex interlocks by crossing over cannot be considered absolutely conclusive, yet it is obvious that the simplest interpretation of the experimental results is that the chromatids fall free of each other (except, of course, at the centromere region) prior to anaphase. It would be tempting to invoke here the explanation advanced by Matsuura ('40) that this is accomplished by separation of chromatids by matrix formation, were it not for the necessity, then, of implying the free occurrence of sister-strand crossing over meiotically in *Drosophila*. The evidence against this seems quite definite, although it might be, that for ring chromosomes, the separation is of a sort that demands even numbers of sister exchanges, which would be genetically undetectable by the usual tests. If this is true, then it is clear that for some unknown reason the simple ring chromosomes in *Drosophila* behave quite differently from those in maize where Schwartz ('53a) has reported a high frequency of double second anaphase bridges originating, presumably, by sister-strand crossing over in a ring chromosome.

#### DISCUSSION

ATWOOD: Cases where you had an excess of single rings arising according to the various explanations that you gave would be reflected in an exactly complementary increase of inviable zygotes. Did you check this?

NOVITSKI: This is not an easy thing to test, for the simple reason that in an attached-X kind of setup at least 50% of the zygotes are inviable. The accuracy with which any frequency of zygote mortality above and beyond this might be determined is so low that it does not appear to be worth

while to try to get information in this particular way. We have made egg counts; the results are extremely variable from one female to the next. This, in combination with a rather low fecundity of compound ring-bearing females, forced us to abandon this approach.

ATWOOD: Are you giving the frequency of excess single rings with respect to the total or only the viable zygotes?

NOVITSKI: It is with respect to the total because at first division there is separation of the compound from its homolog, and, if we mark the homolog so that we can count the number of products of meiosis receiving it, we know that an equal number of products should get the compound or some crossover derivative of it, as the single rings, dicentrics, and things of that sort. So we have a very accurate way of determining the total number of eggs that should have received an X chromosome or some derivative of it, with an accuracy that probably far exceeds anything that we might get by making egg counts.

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# STUDIES ON CROSSING OVER IN MAIZE AND DROSOPHILA <sup>1</sup>

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FOUR FIGURES

It is a general thesis in genetics as well as most other sciences in the biological field that the study of the abnormal is a most fruitful approach by which to gain insight and knowledge into the behavior of the normal. This is especially true in the study of chromosome mechanics such as duplication and crossing over. This paper will deal with some aspects of the problem of chromosome recombination as determined from a study of ring chromosomes in maize and attached-X chromosomes in *Drosophila melanogaster*.

Unfortunately, most of the ring chromosomes which have been found in maize are quite small, being deficient for a large portion of the rod chromosomes from which they were derived. Consequently, they usually occur in trisomic plants which in addition to the ring also carry two normal rod chromosomes. Under these conditions they behave as univalents and show little or no association with the homologous rod chromosomes.

In 1947 a program was launched to induce and isolate ring chromosomes in maize. The procedure followed was to irradiate normal pollen which was used to fertilize plants heterozygous for white or luteus. In this way it was possible to select ring-bearing plants as seedlings in the F<sub>1</sub>. Instability of ring chromosomes results in the loss or change in the ring size, thus seedlings which had obtained the recessive chlorophyll gene through the egg and a ring chromosome through

<sup>1</sup> This work was performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

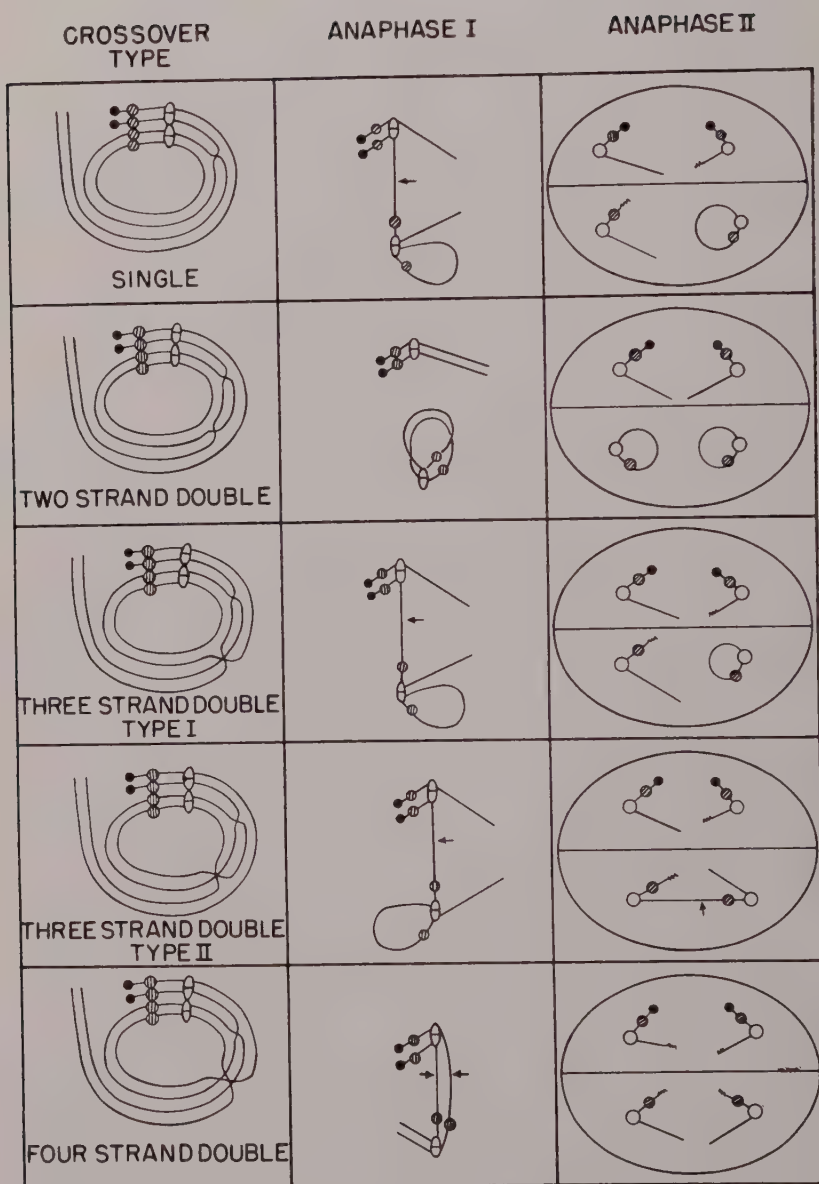


Fig. 1 Anaphase configurations resulting from crossing over between the ring and its homologous rod chromosome.

— = broken chromosome end  
 —→ = position of breakage

the pollen would be mosaic for green and white or luteus tissue. Root-tip sections were examined to verify the presence of a ring chromosome in the striped plants. One of the rings thus isolated involved almost the whole of chromosome 6, the nucleolar organizer chromosome. One of the breaks had occurred near the tip of the long arm, and the other in the strand connecting the satellite with the nucleolar organizer.

The ring is deficient for only a small part of the normal chromosome, and gametes which carry the ring in addition to the other nine chromosomes are viable. Pachytene configurations of ring-rod heterozygotes show close pairing between these two chromosomes throughout most of their length.

TABLE 1

*Meiotic anaphase configurations observed in plants heterozygous for a ring and a rod*

	ANAPHASE I				ANAPHASE II (DAUGHTER CELL PAIRS)			
	Single bridge	Double bridge	No bridge	Total	Single bridge	Double bridge	No bridge	Total
Number	368	81	171	620	166	47	262	475
Percentage	59	13	28	100	35	10	55	100

Single and double bridges are frequently found in both anaphase I and anaphase II. These result from crossing over between the ring and the rod (fig. 1).

As is evident from this figure, the type II 3-strand double and the 4-strand double crossovers result in anaphase configurations which can readily be distinguished, i.e., single bridges in anaphase II and double bridges in anaphase I. A study was therefore initiated to determine the relative frequencies of these anaphase configurations as a method of directly testing for chromatid interference. In the absence of any such interference, the two configurations should occur with equal frequencies. An excess of double bridges in anaphase I would indicate chromatid interference.

The results of these experiments are described in detail in a previous publication (Schwartz, '53). The data (table 1) showed approximately three times as many single bridges

in anaphase II as double bridges in anaphase I. Superficially, this would appear to indicate a negative chromatid interference. However, it was possible to rule this out as the agent responsible for the excess of single bridges in anaphase II.

The discrepancy in the frequencies of the bridge configurations can be accounted for if we postulate the occurrence of sister-strand crossing over. Such a form of crossing over has never really been ruled out. The homozygosis studies with attached-X chromosomes in *Drosophila melanogaster* merely indicated that if sister-strand crossing over occurs it is of a type which does not show the phenomenon of chiasma interference with nonsister-strand crossing over between homologous chromosomes (Beadle and Emerson, '35).

The involvement of sister-strand crossing over does not affect the frequencies of anaphase configurations resulting from the various classes of double crossovers. These configurations, i.e., no bridge anaphase I and II, single bridge anaphase I only, single bridge anaphases I and II, and double bridge anaphase I would occur in a 1:1:1:1 ratio (in the absence of chromatid interference) regardless of the frequency of sister-strand crossing over per bivalent. However, a single crossover between the ring and the rod associated with a sister-strand exchange between the ring chromatids, changes the resulting anaphase configuration from a single bridge in anaphase I only to a single bridge in anaphases I and II. This is shown in figure 2. In other words, the excess of single bridges in anaphase II can be accounted for by sister-strand crossing over in bivalents which had a single nonsister-strand exchange.

From the data it is not possible to establish the frequency of such sister-strand exchanges. All that can be concluded is that the frequency must be high enough for there to be an equal probability of an odd or an even number occurring in the ring. An even number would cancel one another out and give the same result as that obtained without any sister-strand

crossovers. An odd number would in effect behave as if a single exchange had occurred.

If 50% of the bivalents had an effective sister-strand exchange in the ring, it was calculated that 29.5% anaphase II single bridges would be expected: 35% were found. The difference is not statistically significant.

Added support for the assumption of sister-strand crossing over comes from the anaphase II dicentric rings (double bridges). These configurations cannot arise as a result of single or double exchanges between the homologous chromosomes. They result from sister-strand exchanges between the ring chromatids in noncrossover and 2-strand double

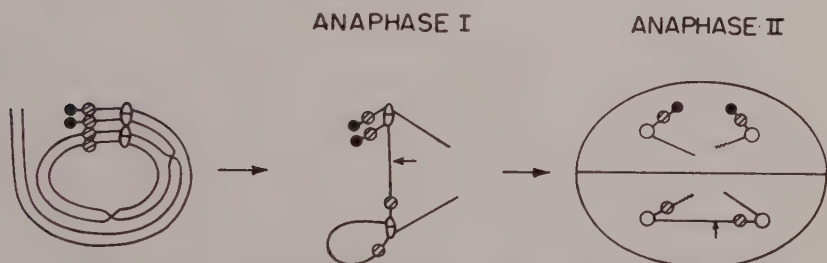


Fig. 2 Anaphase configurations resulting from a single crossover associated with a sister-strand exchange between the ring chromatids.

crossover bivalents. Ten per cent dicentric rings were observed in anaphase II. On the assumption of an equal probability of an odd or even number of sister exchanges, 14% would be expected. The difference between the calculated and observed frequencies is not significant.

In view of the evidence presented for sister-strand crossing over, it is desirable to reconsider the problem of the mechanism of crossing over between homologous chromosomes. The difficulties presented by Darlington's torsion hypothesis have been enumerated a number of times. In fact, any hypothesis which postulates that crossing over occurs through breakage and reunion following duplication of the chromosomes into chromatids is in serious difficulty. It requires that a break in one chromatid should cause a break to occur in exactly the



same position in a chromatid from the homologous chromosome. However, the localization of the crossovers at identical loci offers no difficulty if it is postulated that crossing over is the result of an exchange between the new chromatids during the process of their formation as was proposed by Belling ('31). Such crossing over will result only in the 2-strand double type of multiple crossovers and requires exchanges between sister chromatids to explain the 3- and 4-strand multiple crossover classes (Lindgren and Lindgren, '37). Two crossovers between homologous chromosomes will give rise to a 2-strand double exchange. A sister-strand crossover in one of the chromosomes in the region between the two chiasma will result in a 3-strand double. A sister-strand exchange in each of the homologous chromosomes will result in four single exchange chromatids, a 4-strand double. If the frequency of sister-strand exchanges is high, and there is an equal probability of an even or odd number occurring in this region, the four double crossover classes would appear in a 1:1:1:1 ratio.

A study of somatic crossing over in attached-X chromosomes was undertaken since there is normally little or no sister-strand crossing over in somatic cells of *Drosophila melanogaster*. This is shown by studies on the stability of ring chromosomes in this tissue (Battacharya, '50; Brown and Hannah, '52). The instability associated with such exchanges results from the formation of dicentric rings.

In the absence of sister-strand crossing over, only 2-strand double crossovers would be expected on the hypothesis that crossing over between homologous chromosomes is limited to the newly formed chromatids. Double somatic crossovers are not very frequent, but by using attached-X chromosomes it is possible to determine from single exchanges which strands are involved, i.e., whether or not chromatids which are not attached to a common centromere are involved in crossing over. A more detailed description of these experiments is presented in an earlier publication (Schwartz, '54).

Attached-X flies heterozygous for *y* and *sn*<sup>3</sup>, with the mutants on opposite chromosome arms, were used in this study. These are the same markers which Stern used in his classical study of somatic crossing over between free-X chromosomes. Scoring was limited to twin spots of *yellow* and *singed* tissue since this is the only class of spotting which can only result from somatic crossing over. Spots of *yellow* or *singed* alone can also arise from mutation or chromosome breakage and loss.

Heterozygous attached-X females of the constitution *Hw y sn<sup>+</sup>·sn y<sup>+</sup> Hw<sup>+</sup>* were mated to M-5 males. By scoring only those offspring which were *hairy wing*, *non-yellow*, and *non-singed* it was possible to limit the scoring to flies of the genotypes *y sn<sup>+</sup>·sn<sup>+</sup> y<sup>+</sup>*, *y sn·sn<sup>+</sup> y<sup>+</sup>*, and *y sn<sup>+</sup>·sn y<sup>+</sup>*. Only the last of these can give twin spots through crossing over and is of the desired genotype. Since it was not possible to distinguish between these three classes without progeny tests, a correction factor was needed to correct for those flies in the scored population which could not give twin spots.

The relative frequencies of these three genotypes in the population were determined from table 2 of the Beadle and Emerson paper ('35). They used the markers *scute* and *cut* which are in approximately the same positions as *yellow* and *singed* (*sc* and *y* are both at 0.0, *ct* is at 20.0, and *sn* is at 21.0). From this table it was calculated that 76.5% of the flies scored were of the desired constitution.

Stern ('36) found that twin spots arise from crossing over in heterozygous flies carrying two free-X chromosomes. With free-X's twin spots can result from crossing over between any of the chromatids, depending on the type of segregation (X or Z—see Stern, '36). However, with attached-X chromosomes twin spots can result only from crossing over between a new and an old strand (fig. 3A). If crossing over occurs at random between any of the four chromatids twin spots should result. On the other hand, if crossing over is limited to the new chromatids no twin spots should be found (fig. 3B). Twin spotting in the attached-X flies might be expected to

be even more frequent than in free-X females since the attachment of the chromosomes at one end could make for closer somatic pairing. The data are shown in table 2 (lines 1 and 2). The autosomal minute,  $M(3)y$ , was used to increase the somatic

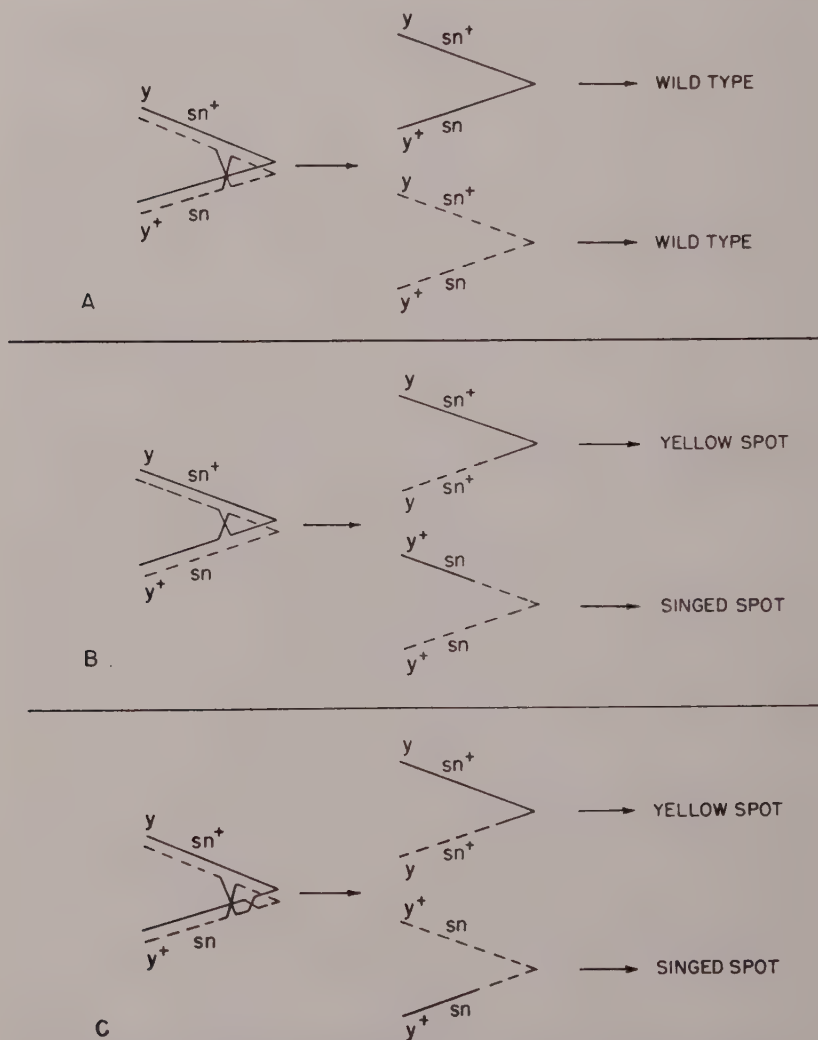


Fig. 3 Diagrammatic representation of the results of somatic crossing over in attached-X chromosomes. Broken lines represent the new chromatids. (A) and (B) not associated and (C) associated with a sister-strand exchange.

crossover frequency. It is readily apparent from this table that the frequency of twin spotting in the attached-X material is extremely low as compared to that obtained when the same markers were used in free-X chromosomes. This indicates that crossing over between homologous chromosomes in attached-X's is limited to the newly formed chromatids. The few cases of twin spots observed in the attached-X experiments could be caused either by a low frequency of sister-strand crossing over (to be discussed) or by a breakdown of the attached-X's followed by somatic crossing over.

Brown and Hannah ('52) have reported that aging of females as virgins before mating was responsible for a high degree of instability, in the offspring, of ring chromosomes

TABLE 2

*Frequency of twin spotting in females heterozygous for y and sn  
(mutant genes located in opposite chromosome arms)*

	TOTAL FLIES SCORED	CORRECTED <sup>a</sup> TOTAL	NUMBER OF TWIN SPOTS	PERCENTAGE OF TWIN SPOTS
1. Attached-X	1360	1041	8	0.77
2. Attached-X (aged)	926	709	48	6.77
3. Free-X	376		53	14.09

<sup>a</sup> See text.

in somatic tissue of *Drosophila melanogaster*. One hypothesis proposed to explain this effect was that aging increased the frequency of sister-strand crossing over. On the basis of this report the effect of aging on twin spotting in attached-X flies was studied.

On the hypothesis that crossing over between homologous chromosomes is limited to the new chromatids, twin spotting should result if in addition sister-strand crossing over occurs. A crossover between the new chromatids associated with a proximal sister-strand exchange will result in a twin spot (fig. 3C). However, if crossing over occurs at random between any of the four chromatids, the frequency of twin spotting should not be altered by sister-strand crossing over. A sister exchange will cause a twin spot to be formed when



associated with a crossover between chromatids which are attached to a common centromere, but conversely, only wild-type tissue will result when the exchange is associated with a crossover between chromatids which are attached to different centromeres. The results of the aging experiments are given in line 3 of table 2. Aging was responsible for an 8.7-fold increase in twin spotting in attached-X flies. That the effect of aging was not to cause an over-all increase in the amount of somatic crossing over is evident from the fact that no difference was observed in the frequency of twin spotting in the free-X females from aged and unaged mothers.

The results of these experiments suggest that chromosome duplication involves the formation of a new chromatid on a template provided by the parent chromosome. If duplication involved the splitting of a double-sized chromosome into two equal chromatids, both of these should have been equally capable of participating in nonsister-strand crossing over. These experiments further suggest that somatic crossing over is limited to exchanges between the new chromatids. It is proposed that meiotic crossing over involves both exchanges between the newly formed chromatids and between sister chromatids.

To date, all the studies relating to the mechanism of crossing over have been most indirect. From genetic and cytological recombination studies we have attempted to draw conclusions as to the behavior of an entity about which we know very little. What is a chromosome? We can describe it quite well from a genetic or cytological point of view. However, this is not enough if we are concerned with such problems as the mechanism of chromosome replication and recombination. Critical studies of these processes will be possible only when more information is available on the molecular structure of the chromosome.

It is for this reason that the work of Watson and Crick ('53a, b, c) on the structure of deoxyribonucleic acid (DNA) and its possible relation to the gene is so exciting. Here is a model for a gene. Can we now go one step further and



tie this gene in with the chromosome in such a fashion as to fit the cytogenetic picture of chromosome mechanics?

I should like to present a *cytologist's* attempt to construct a model of the chromosome based on the Watson-Crick model for DNA. I fully realize that this is in the realm of pure

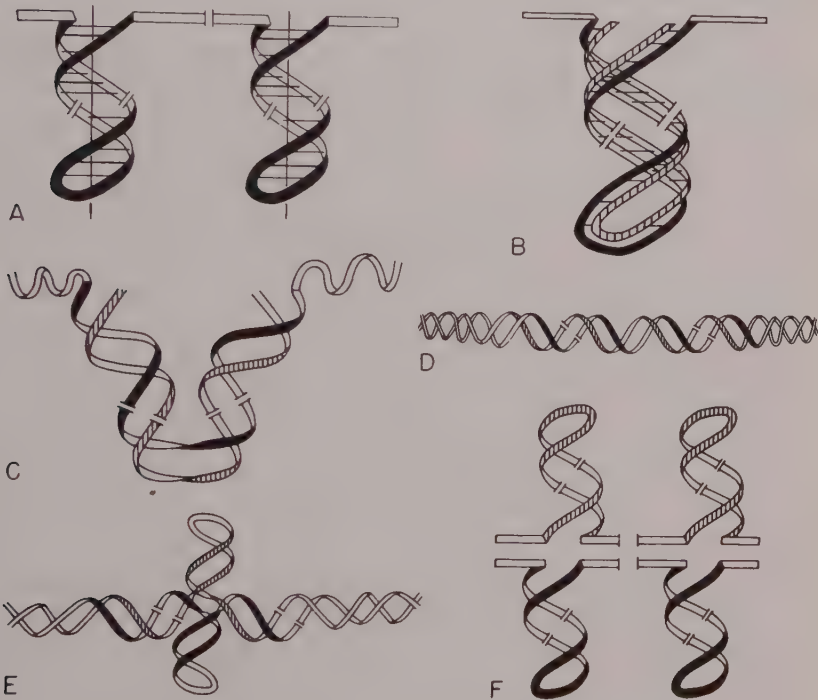


Fig. 4 A schematic diagram of the duplication of the proposed model of a chromosome. The solid ribbons represent the old and the crosshatched ribbons the new DNA molecules. The completely unshaded ribbons are proteins.

speculation. However, I believe that such attempts are important and should be made, since the Watson-Crick DNA model is of little value to the cytogeneticist unless it can be incorporated into a chromosome.

The proposed model of the chromosome is diagrammed in figure 4A. The chromosome is considered as being composed of alternate sections of protein and DNA linked up in the

fashion indicated in the figure. The DNA structure as shown is that proposed by Watson and Crick except that the strands at one end are bonded together. In other words, the Watson-Crick model is considered as being a single strand which is coiled back on itself forming a doubled structure. This is essential for chromosome continuity. The width of the chromosome is thus equal to the length of the DNA molecule, and is hence in the range of microscopic visibility. At this point the DNA is plectonemically coiled in a right-handed direction and the protein is uncoiled.

The first stage in the duplication and separation of the chromosome is the duplication of the DNA (fig. 4B). The protein is still single. The original hydrogen bonds are broken and reformed in the new DNA molecules as described by Watson and Crick. The two models are still right-handedly plectonemically coiled around each other.

The next step involves the coiling of the protein in a left-handed direction. This will cause the two DNA models to uncoil, still keeping the coiled doubled structure of each model intact (fig. 4C). At this point the protein is duplicated (fig. 4D). Since the original protein strand was left-handedly coiled, the two strands after duplication will be plectonemically coiled in a left-handed direction. Separation is facilitated since the left-handed coils in the protein will compensate for the right-handed coils in the DNA. The uncoiling of the protein will uncoil the two DNA strands and each of these will coil back on itself as is shown in figure 4E. At the completion of this process we are back to the point at which we started except that there are now two separate chromosomes (fig. 4F). One is the intact old and the other the new. According to the model, crossing over will result when adjacent new DNA molecules, one from each of the homologous paired chromosomes, are linked together by the new protein strand.

It should be noted that it is not necessary to assume a template mechanism for the duplication of the interstitial protein, nor is it necessary to have different specificities or

any linear differentiation in the protein itself. That is, the same protein could be inserted at all levels along the chromosome, and could be synthesized somewhere else in the cell. This removes the objection that proteins are not supposed to have self-replicative properties.

In conclusion, this model represents an attempt to construct a chromosome composed of protein and DNA (based on the Watson-Crick model) in such a fashion that duplication and separation can be accomplished without the chromosome being broken up into many small fragments.

#### DISCUSSION

CRICK: As I understand Dr. Schwartz' structure, one of its features is that when the new protein strand is made it has to be coiled plectonemically in the opposite sense to the DNA. It seems to me that unless the net number of plectonemic terms (summed, taking account of sign, for all parts of the structure) is zero, the two parts of the structure cannot come apart unless of course there is further untwisting of the two ends.

SCHWARTZ: This model requires a correlation between the number of coils in the DNA and in the protein-connecting strands. In other words, the amount of coiling in the protein is conditioned by the coiling present in the adjacent DNA molecules. This could be accomplished by having the two DNA models uncoil during the process of their duplication, thus putting left-handed coils in the connecting protein strands. Many such modifications of this system are possible without changing the basic concept of the model.

CRICK: I would just make the point that it is not structurally plausible to add two extra polynucleotide chains to the present DNA model, as shown in Dr. Schwartz' slide, though it is just possible that the same effect could be gotten in some slightly different way. The point about our DNA model is that it is a fairly precise one. We really have no comparable model for the protein part of nucleoprotein.

In general, I think it is true to say that, chemically, it is quite easy to conceive of protein branching. The reason is that some of the side chains of the protein are such that they can easily tack onto the main chain. On the other hand, DNA is a structure which one thinks would have very considerable difficulty in branching.

It does seem to me that one of the most striking facts of genetics is that there is little or no branching of the chromosomes. This could be due to biological reasons, but I would have thought it had a molecular basis. This would suggest to me that the molecular basis of the chromosome must be one long, unbranched chain; and there are chemical reasons — I wouldn't say good, but fairly good ones — for pointing a finger at DNA if one has to look for something of that sort.

So I think there is a certain small amount of evidence in favor of models in which the DNA runs continuously, or potentially can run continuously, right through from one end to the other. This leads us immediately into very great difficulties in unwinding such a structure. I think the answer will be in some sort of breaks, which was mentioned earlier. In my opinion, one of the most fruitful lines of research is to study whether breaks occur in the isolated material, and also whether they occur in intact biological material.

Another general point is that one is appalled at one's lack of knowledge immediately upon getting onto a slightly larger scale — there is obviously structure in the chromosome on this scale, and there is protein associated with this structure. I would very much like to know what the molecular structure looks like at a resolution of, let's say, 100 Å. I think the electron microscopy of chromosome structures is being neglected and perhaps might be taken up again.

Whether, from the purely genetic side, the formal schemes which have been proposed will give any information about the molecular structure, i.e., whether the two will tie up, is very difficult to say. As I understand it, there is no really agreed formal scheme. If I ask a geneticist what it is that I have to explain, I find I do not get a clear answer. It is



difficult, therefore, to try to make a synthesis at this particular stage, although one is certainly tempted to try.

STURTEVANT: I share with Dr. Novitski the dislike of having the *Drosophila* ring specified as an unusual one. I gather, Dr. Schwartz, that your ring was selected in the first instance because of its instability—I think that in *Drosophila* it would be possible to select an unstable one. I would suggest that perhaps yours is the unusual type of ring.

I would also like to inquire whether, in the comparison of the twin spot frequencies for the free X's and the attached X's, you took into account Y chromosome? Did they differ with respect to the Y chromosomes present?

SCHWARTZ: The free-X females did not carry a Y chromosome; the attached-X females did. However, Stern has shown that the effect of the Y is to increase somatic crossing over rather than decrease it.

LEDERBERG: I suppose all of us are troubled by the problem of breakage, especially simultaneous breakage of two chromatids, in crossing-over theories and for this reason are interested in alternative proposals. But is that not the mechanism you propose for sister-strand crossing over, or do you have something else in mind? Am I right that the theory still calls for a breakage for the sister strands and a new mechanism for the homologous strands? There is still a physical breakage?

SCHWARTZ: It is impossible to explain 3- and 4-strand doubles on any hypothesis without breakage of one or both of the parental strands. The difficulty lies in the localization of the breaks at identical loci in the chromatids involved in a crossover. Going back to the model, one can get around this difficulty by assuming that sister-strand crossing over occurs at the stage when the DNA is doubled but the protein is still single. If a break occurs at the point of connection of the protein and the DNA, and the protein strand then re-joins with the other (new) DNA molecule, a sister-strand exchange will result.



GALL: You have objected that the Belling hypothesis does not permit 3- and 4-strand double exchanges. In his 1933 paper, Belling himself shows how these could occur. I wonder if your results could be explained by assuming a rotation of the cleavage plane between chiasmata. In that case you would need to postulate no breakage at all.

SCHWARTZ: This is essentially a sister-strand exchange. My objection to this hypothesis is that it assumes that chromosome duplication involves the splitting of a double-size chromosome rather than the formation of a new chromatid on a template mechanism. I feel that the evidence presented here on somatic crossing over rules this out.

SHULT: In the rod-to-ring synapsis, the evidence for sister-strand exchange rests on the high frequency of double bridges observed at anaphase II. It was assumed by Dr. Schwartz that these were formed by ring-to-ring sister-strand exchanges in spite of the fact that they might be explained equally well on the basis of nonsister exchanges.

For example, one-sixteenth of the triple crossovers will produce a double bridge at anaphase II. However, as the number of crossovers is increased, the expected frequency of double-bridge formation approaches one-twelfth, thus reducing the significance attached to the observed frequency. For this reason, in the absence of precise knowledge concerning the average number of crossovers occurring over the entire length of the chromosome per meiosis, it is impossible to draw any conclusion regarding the amount of sister-strand exchange.

SCHWARTZ: Five exchanges per bivalent are required before the frequency of AII double bridges becomes one-twelfth, in the absence of sister-strand crossing over. Crossing-over frequencies of this order are much too high for maize and do not agree with the rest of the data. It was the combination of both a high frequency of double bridges in AII and the excess of single AII over double AI bridges which forced the conclusion that sister-strand exchanges must be involved.

GLASS: I am rather troubled by your postulate of an equal probability of a sister-strand exchange occurring or

not occurring in the region between the nonsister exchanges. This seems to me not to be plausible in terms of conventional ideas of interference between exchanges, for it disregards the fact that ordinary exchanges exhibit interference inversely as the distance between the exchange points. Consequently, I would expect that sister-strand exchanges would vary in probability with the extent of the distance between the nonsister chiasmata, i.e., would be more likely to occur if the distance was great and less likely if the distance was small. This would, in fact, follow even if there was no interference at all, but simply a probability of exchange proportional to chromosomal length. How then can you postulate that there will be "equal probability of a sister-strand exchange occurring or not occurring in this region," irrespective of the length of the region? That would imply some very special and fancy mechanisms.

SCHWARTZ: If the number of sister-strand exchanges per chromosome is high and they show a Poisson distribution, an equal probability of an odd or even number of sister-strand exchanges between the chiasma would be expected except for the very small regions. Any region having a mean of two or more sister-strand exchanges per chromosome should show about an equal probability of an odd or even number of exchanges and hence no chromatid interference. For the small regions having a mean number of less than 1 or 2, there would be an excess of the even number of exchanges, and thus an excess of 2-strand doubles would be expected. The attached-X studies in *Drosophila* seem to bear this out.

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# CROSSOVER VARIABILITY AND INDUCED CROSSING OVER <sup>1</sup>

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SEVEN FIGURES

A distinction has been indicated in the title between cross-overs and crossing over. The former may be observed directly as the recombination fraction of the offspring, whereas the latter is a process resulting in exchange between homologs. Crossing over is always an inference from the breeding data. In much of the early work in genetics the assumption has been made that recovered crossovers arise independently of one another. However, a large body of data reveals a variable relation between the products and the process of crossing over. The variability in data on spontaneous as well as on induced recombination values in *Drosophila* may be explained by assuming the occurrence of a small amount of gonial crossing over.

One of the first environmental-genetic interactions to be discovered was the effect of high or low temperatures of long duration on recombination values (Plough, '17). Since that time other agents, such as ionizing irradiation and certain chemicals, have been found to affect recombination and mutation rates and to alter phenotypic expression. These three phenomena are not always easy for the experimenter to sort out, one from another.

The main problem of this paper is the identification of the mechanism and location of induced crossing over, dependence being primarily on genetic data from *Drosophila*, since cyto-

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logical studies in this organism are difficult. Furthermore, Cooper ('49) has pointed out a significant lack of correlation between chiasmata in untreated *Drosophila* males and genetic recombination. Hence there seems to be a three-way hiatus in cytogenetics. One chiasma does not correspond to one crossing over; and one crossing over does not delimit the number of descendant crossover chromosomes classified at some later time. Hence the crossover data cannot be used with confidence to predict what frequency of chiasmata might

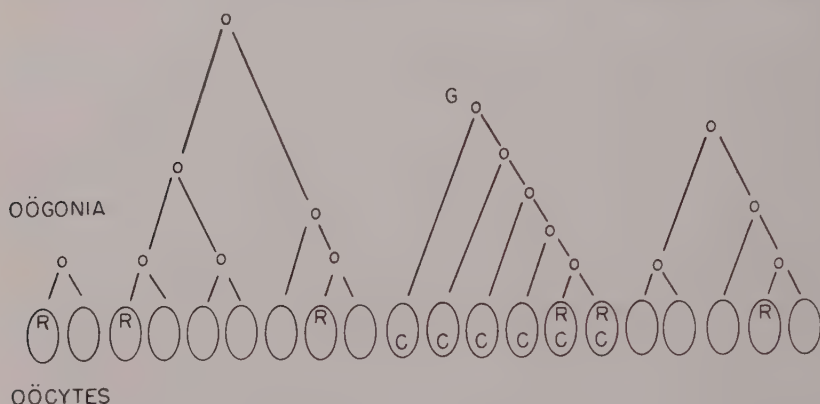


Fig. 1 Three ways of obtaining a given recombination value by different amounts and kinds of crossing over.

R = Random meiotic  $\rightarrow 3/20$

G = Gonial (mitotic)  $\rightarrow 3/20$

C = Conditioned meiotic  $\rightarrow 3/20$

be observable. The question is raised here as to whether the *Drosophila* results do or do not apply to other organisms. Figure 1 illustrates various ways in which crossing over may occur, and it will be shown that two of them are not confirmed by critical data on crossover distributions in *Drosophila*.

If a body of tester-cross data consists of a recombination fraction, e.g., 15%, at least three modes of origin may be inferred. The simplest and oldest assumption is that crossing over occurred in six or so primary oöcytes independently of each other. Such random meiotic crossing over in some section of the chromosome of moderate length might be represented



as occurring in the large oöcytes marked R (in figure 1). For this sample of random and independent crossovers the calculation of a sampling error of 8% would be correct. Random meiotic crossing over would perhaps remain the only assumption considered today, but for certain evidence from irradiated *Drosophila* presented or reviewed in this paper.

A second way of obtaining the same recombination frequency, 15%, involves three steps. (1) In some gonial cell, G (see figure 1), a chromosome might be weakened at a certain point, either by irradiation or spontaneously. (2) This chromosome and its weak point might be multiplied and handed on to a small or large number of primary gametocytes. (3) During meiosis each of the chromosome tetrads from the common source in cell G might undergo crossing over at the weakened point while no gametocytes would form other crossovers. This possible sequence may be named "conditioned crossing over," meaning gonial predisposition to subsequent meiotic exchanges in identical regions. It could as easily provide a sample of 3/20 crossovers as 0/20 crossovers, depending on the occurrence or nonoccurrence of weakening in cell G. Therefore, the appropriate standard error would be larger than that previously stated for three crossovers formed independently in a sample of twenty.

A third possible inference of the origin of the same 15% sample of recombinations is the induction and completion of crossing over in just one gonial cell. From the one gonial cell, G in figure 1, crossover chromosomes would pass to the cells, C, to half of the sperm or eggs. Thus gonial crossing over would produce a nonrandom distribution of crossover progeny similar to "conditioned meiotic crossing over," a distribution which could fluctuate widely depending on the amount of gonial multiplication. If a different cell had been the site of gonial conditioning, or of completed crossing over, a much larger or a much smaller number of recombinations would have been expected in the test cross data.

Although superficially alike in their nonrandom total frequencies, gonial crossing over and gonial conditioning may

be separated by comparison of the pairs of complementary classes. Complementary classes would be as equal after conditioned meiotic as after random meiotic exchanges; but complementary classes could vary tremendously whenever crossover chromatids segregate in gonial cells which then divide many times.

Consideration of the problem will proceed from its general to its special aspects. The best known situation is the change in the amount of crossovers from irradiated normal *Drosophila* females, from which it has frequently been inferred that irradiation increases or decreases crossing over at meiosis depending on proximity to or distance from the centromere. The second group of experiments will deal almost entirely with induced recombination, by use of specimens where spontaneous crossing over is naturally rare or is inhibited by inversions. The third group of experiments will involve lethals opposite isogenic wild chromosomes in order to discriminate between conditioned meiotic crossing over and completed gonial crossing over. Models for gonial crossing over and subsequent multiplication of crossover and non-crossover chromosomes may be found in somatic twin spots as analyzed by Stern ('36) and by Brink and Nilan ('52).

#### CHANGES IN RECOMBINATIONS FOLLOWING IRRADIATION OF HETEROZYGOUS "RUCUCA" FEMALES

Mavor and Svenson ('24a, b) found that irradiation increased recombination values in chromosome 2, but not in the parts of the X chromosome far removed from the spindle attachment (Mavor, '23). Muller ('25) demonstrated that X irradiation affected recombination frequencies differentially along the long autosomes in *Drosophila* females, the greatest increase being at the centromere. Plough ('24), using  $\gamma$  rays of radium, found alteration of linkage values similar to those reported by Mavor and Svenson for X rays. Whittinghill ('51), using  $\text{Co}^{60}$   $\gamma$  rays, obtained results which are plotted in figure 2.

The third chromosome multiple recessive stock "rucuca" (*ru*, roughoid; *h*, hairy; *th*, thread; *st*, scarlet; *cu*, curled; *sr*, stripe; *e<sup>s</sup>*, sooty; *ca*, claret) was used; figure 2 gives loci on the standard chromosome map. Sampling variation is indicated by the vertical width of each black band, which represents one standard error of the ratio of the difference between the crossover values of treated and control series. The increase in the spindle attachment region, *st* to *cu*, was  $2\frac{1}{3}$  times the control value for that region. Near this the increases were less pronounced, but the *cu* to *sr* region also showed significantly more crossovers from the  $\gamma$ -irradiated

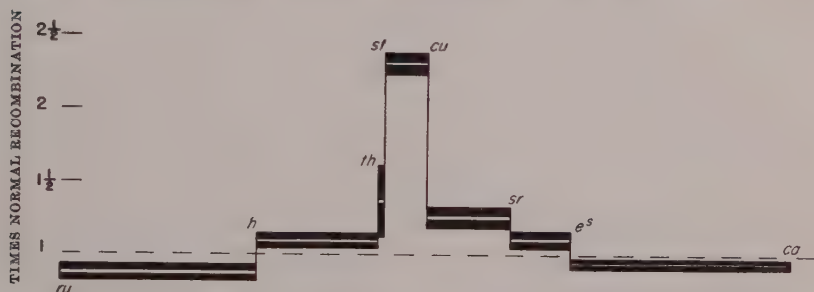


Fig. 2 Ratio of recombination values from irradiated and from control females, for 20 days of breeding after treatment of adult *Drosophila melanogaster* females with 4000 r  $\gamma$  rays at 8.33 r/min.

females. The next most distal regions in each chromosome arm did not show significant changes; but the terminal regions showed less recombination in the  $\gamma$ -irradiated series than did the control series; and the depression in the *ru* to *h* region was statistically significant, as computed on the assumption of completely random meiotic exchanges. The alterations in the irradiated series are thus graded from a maximum increase at the spindle region to normal or subnormal in the most distal regions of the chromosome. This pattern of change had previously been observed following other treatments and was named by Kikkawa ('34) "the proximal increase and compensatory distal decrease." Although the evidence from whole experiments followed this pattern, there seemed to be

some nonrandom distributions from family to family as have already been discussed (Whittinghill, '51). These slightly nonrandom crossover variations from ordinary females may perhaps be explained by the study of crossover induction in other material where the background of spontaneous crossing over is low or absent. Experiments with *Drosophila* males, or with females homozygous for the asynaptic factor *c3G* (Whittinghill, '37, '38) or with females heterozygous for inversions which effectively eliminate most normal crossovers are useful for further studies.

#### NONRANDOM RECOMBINATIONS INDUCED BY IRRADIATION

The first published data to be attributed to spermatogonial crossing over were the highly nonrandom recombinants in experiments by Friesen ('36). Table 1 contains a rearranged summary of his testercrosses of 60 X-irradiated males. Ten to sixteen days after treatment, when recombinations were at a maximum following a 4000-r dose, the males produced 18,152 testercross offspring. Crossovers comprised 3.2% of these, but they were concentrated in a few families. Forty-four families contained no crossovers, although they had the majority of the offspring. Five families containing one to five crossovers have been grouped, and they, not surprisingly, show a low frequency of recombinants. Those with more than five have been presented individually, arranged in increasing order of numbers of crossovers produced. Recombinants were 13, 14, 16, 22, 27, and 48% in these six families of the fifty-six fertile males in spite of the low over-all expectation of 3%. Within the families shown here, it will be noted that the crossovers most often resulted from exchange in a preferred region, usually between *st* and *cu*, but also between *ru* and *h* or between *sr* and *e*<sup>s</sup> in some families within the grouped data. Occasionally a male produced crossovers showing that single exchanges had taken place in more than one region.

By contrast with these males, normal crossing over in a heterozygous rucua female having several hundred offspring would form recombinations involving crossing over in all regions, with the possible exception of the shortest *th-st*

TABLE 1

*Offspring from sixty Drosophila males testcrossed 10-16 days after X irradiation.  
(Data of Friesen, '36, table 1, rearranged)*

FAMILIES	NONCROSSEOVERS	CROSSEOVER FLIES	PERCENTAGE CROSSEOVERS
1-44	14,084	None	0
		3 <i>ru</i>	
		1 <i>h th st cu sr e<sup>s</sup> ca</i>	
45-49	1,840	1 <i>ru h th st</i>	0.7
		5 <i>cu sr e<sup>s</sup> ca</i>	
		1 <i>ru h th st cu sr</i>	
		2 <i>e<sup>s</sup> ca</i>	
50	391	11 <i>cu sr e<sup>s</sup> ca</i>	2.7
51	251	23 <i>ru h th st</i>	13.0
		14 <i>cu sr e<sup>s</sup> ca</i>	
52	302	32 <i>ru h th st</i>	14.0
		16 <i>cu sr e<sup>s</sup> ca</i>	
53	298	49 <i>ru h th st</i>	22.0
		37 <i>cu sr e<sup>s</sup> ca</i>	
54	245	50 <i>ru h th st</i>	27.0
		42 <i>cu sr e<sup>s</sup> ca</i>	
55	505	49 <i>ru h th st</i>	16.0
		47 <i>cu sr e<sup>s</sup> ca</i>	
56	236	120 <i>ru h th st</i>	48.0
		105 <i>cu sr e<sup>s</sup> ca</i>	
Total	18,152	608	3.2

region. Furthermore, some of these offspring from normal females would be double, triple, and perhaps quadruple cross-overs. Thus this sample of Friesen's data, which is confirmed by other data some of which will be cited, is only remotely like meiotic crossing over and resembles what would be expected from rare gonial crossing over or else from the rare



weakening of a gonial chromosome in some one region. Friesen's data as published come just short of allowing a test for this distinction. For instance, additional information could be used on the remaining flies of the family which had eleven *cu sr e<sup>s</sup> ca* flies and none of the complementary class, or on the 32:16 distribution.

If crossing over occurs only at meiosis, the recovery of crossover strands would represent a series of 1-strand samples from different crossover tetrads, and the numbers of complementaries within families should be distributed as randomly as are males and females within sibships. Gonial crossing over resulting in the inclusion of complementary crossover chromosomes in different daughter cells might result in clusters in which the two complementary classes differed greatly in size, if the rate of multiplication of the two daughter cells should differ. A distinction may be made by summarizing the ratios from each family.

The data from the large crossover producers of the previous experiment have been combined with data from a similar and larger experiment of Friesen's ('36), and the pairs of complementary crossovers have been arranged in table 2 from most probable to least probable. In this presentation equality of recovery has been assumed. Although there is evidence from Friesen's total of 747:556 that the *ru h th st* class survives better than its complementary, calculation of probabilities of deviating as far or farther from a 57:43 expectation merely changes the order of a few of these pairs of classes but still leave an excess of improbable families. Sufficient viability data for classes resulting from the less numerous crossovers away from the spindle attachment were not available from Friesen's experiment, so again the probability of each sampling deviation has been calculated from an expectation of 50:50. This was an added reason for presenting *P* for the spindle region on the same basis.

In ranking samples from closest to poorest fit, as in table 2, it would be expected, by definition, that half of the entries would lie above the 50% probability level and half below it.

However, the median for the twenty pairs of complementary crossovers for the spindle region is near the 10% level. There are more ratios below the 1% level than there are above the 50% level. In the more distal regions combined there is also an excess of complementaries which would be unlikely on the basis of late recombination. The median of eleven clusters of distal crossovers is below the 1% probability level.

TABLE 2

*Numbers of flies in complementary crossover classes compared with 50:50 expectation. (Data from Friesen, '36, tables 1 and 2)*

<i>ru h th st:cu sr e<sup>2</sup> ca</i>	OTHER PAIRS	<i>P</i>
13:12	49:47	1.00
4:4		
2:3	3:5	
3:5	2:4	0.40
50:42		
4:8		
120:105		
190:166		0.20
49:37		
23:14		
82:59	Median	0.10
19:35		
32:16	0:7	
163:122	31:14	0.01
62:30	41:77	Median
43:17	0:9	
0:11	57:20	
0:21	0:18	
0:32	27:0	
140:28	27:201	

The one-sided ratios do not appear to be due to viability differences, but rather to a mechanism readily producing wide dispersions. The extreme ratios in the bottom parts of each column of table 2 favor one class as often as the other complementary class. Thus these X-ray-induced recombinations are genuinely nonrandom in respect to complementary classes as well as being agglutinated as to region of the chromosome and family of origin. Although meiosis could only remotely produce such a distribution as that in table 2, a simple explanation is available, i.e., that only thirty-one occurrences of crossing over produced over two thousand recombination

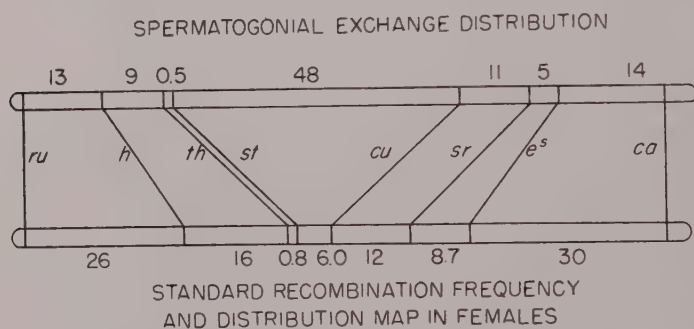


Fig. 3 Comparison of the gonial map based on data from irradiated males with the standard female map of the third chromosome of *Drosophila melanogaster*.

offspring confined to thirty-one "spots" as to familial and regional location.

With such conservative estimates the distribution along the chromosome of the minimum diagnosed crossings over in the male may be compared with the distribution of spontaneous recombination eggs of *Drosophila* females. Figure 3 contains such a comparison.

The spermatogonial map has been constructed from the data of Friesen ('36) and Whittinghill ('47, '48), where a minimum of 189 occurrences of crossing over are necessary to account for the crossover distribution found. The inference of thirty-one of these emerges directly from table 2. It should be noted that the spermatogonial map does not repre-

sent frequency but only the spatial distribution of those rare crossings over which result from irradiation. Almost half of the total spermatogonial crossings over occurred in the *st-cu* (centromere) region, a region with less than 6% recombination in the female. It will be recalled that this region is also the one in which is found the greatest increase in cross-over values in irradiated females.

When the distribution of recovered crossover chromosomes was examined in the same data, 70% of all induced crossover offspring were found to be recombinations for the spindle attachment region. Data of Parker ('48) also show 60–70% of observed recombinations localized in the spindle attachment region. It is possible that a similar distribution of oögonially produced crossovers may have been added to spontaneous meiotic crossovers in females to produce the changes in total crossovers of all kinds, as reflected in figure 2.

Some  $\gamma$ -ray experiments on males of *Drosophila* differed from those heretofore performed. A crossover selector technique (Whittinghill, '50) used linked recessive lethals in such a way that all noncrossover zygotes would die (table 3). Only one of the two complementary classes resulting from crossing over in the male would regularly survive. This allows testing of environmental agents for ability to induce crossing over in males. It is possible to see if there is a random or a clustered familial distribution, but a comparison of complementary classes cannot be made.

In the  $\gamma$ -ray experiment each irradiated *My Gl/Sb bx<sup>p</sup>* male was placed with several *My Sb/Gl bx<sup>p</sup>* females in a separate vial. These flies were transferred to fresh vials, and additional females were added on the sixth, tenth, and sixteenth days. The first two sets of vials had very few offspring scattered among the families, and the phenotypes of these early survivors indicated spontaneous crossing over in the short flanking regions of the untreated females. After the tenth day from irradiation, some of the males produced a few more live offspring, which usually indicated crossing over in the spindle attachment region of the  $\gamma$ -irradiated fathers.

These crossover sperm had fertilized eggs of the various kinds in the proportions expected from the standard map distances, so that rough estimates of the total numbers of eggs laid could be made as described in an earlier publication (Whittinghill, '51).

For the present purposes, the distribution of crossovers per family is of interest (table 4). There were fifty-nine

TABLE 3

*Genotypes of surviving adults from the lethal system of crossover-selector matings:*

<i>My</i>		<i>Sb</i>		females by treated	<i>My Gl</i>		males
<i>Gl</i>		<i>bx<sup>D</sup></i>			<i>Sb bx<sup>D</sup></i>		
EGGS: FREQUENCY AND KIND				SPERMS			
				Noncrossovers		Crossovers, if any	
				0.50- <i>My Gl</i>	0.50- <i>Sb bx<sup>D</sup></i>	<i>My Gl Sb bx<sup>D</sup></i>	+
0.41	<i>My</i>	<i>Sb</i>					<i>My Sb/+</i>
0.41	<i>Gl</i>	<i>bx<sup>D</sup></i>					<i>Gl bx<sup>D</sup>/+</i>
0.08	<i>My</i>	<i>bx<sup>D</sup></i>					<i>My bx<sup>D</sup>/+</i>
0.08	<i>Gl</i>	<i>Sb</i>					<i>Gl Sb/+</i>
All these combinations die except —							
0.006	<i>My Gl</i>	<i>bx<sup>D</sup></i>					<i>My Gl bx<sup>D</sup>/+</i>
0.006		<i>Sb</i>		<i>My Gl/Sb</i>			<i>Sb/+</i>
0.003	<i>My</i>	<i>Sb bx<sup>D</sup></i>			and		<i>My Sb bx<sup>D</sup>/+</i>
0.003	<i>Gl</i>				<i>Gl/Sb bx<sup>D</sup></i>		<i>Gl/+</i>
Fate of zygotes, by sperm classes				0.994 die	0.997 die	All die	All survive, if formed.

fertile males of suitable constitution, fertile to the extent of producing larvae. In the vast majority of families these larvae never gave rise to adult flies because of the linkage arrangement of some of the later-acting lethals. If any male did have crossovers, he seemed able to have many about as easily as few recombination offspring. Thus the fourteen responding males had crossovers to the number of 1, 2, 4, 6, 7, 9, 12, and 21. This was a non-Poisson spread, whereas their females were having spontaneous crossovers mostly 1, 2, or 3 per



group of females, a distribution which remains in accord with meiotic expectation.

Thus  $\gamma$  rays as well as X rays have produced crossovers in *Drosophila* males where none are expected spontaneously. Their distribution has been so nonrandom that they may be attributed to gonial crossing over. However, the question remains whether X and  $\gamma$  rays and other inducing agents act the same way in females as they do in males. This problem has been investigated by using females which do not without treatment produce any appreciable number of crossovers.

TABLE 4

*Total numbers of fertile matings producing 0, 1, 2, or more live adults.*

Single *My Gl*  $++/++$  *Sb bx<sup>D</sup>* males irradiated with 4000 r of  $\gamma$  rays were mated to many *My*  $+$  *Sb*  $+/+$  *Gl*  $+$  *bx<sup>D</sup>* (or to *My*  $++$  *bx<sup>D</sup>/+* *Gl Sb*  $+$ ) females.

All live offspring are crossovers in this system of mating.

SOURCE OF CROSSOVERS	TOTAL CROSSOVERS PER SINGLE MALE TESTED												TOTAL FERTILE FAMILIES
	0	1	2	3	4	5	6	7	8	9	12	21	
Induced in males in region 2	45	4	3	1	1	—	1	1	—	1	1	1	59
Spontaneous in females in													
region 1	46	15	5	—	—	<sup>a</sup>	—	—	—	—	—	—	66
region 3	49	13	3	1	—	<sup>a</sup>	—	—	—	—	—	—	66

<sup>a</sup> Represents one family omitted from the table which had 5 offspring classified as *My Gl Sb bx<sup>D</sup>* and 20 classified as *My Gl Sb*  $+$ , which could represent region 1-3 double crossovers and region 1 singles, respectively.

Earlier experiments of the author ('38) on crossing over in the sex chromosomes of *c3G* asynaptic females indicated the existence of oögonial crossing over, but later experiments on structurally heterozygous females have provided more adequate data.

The next experiments will be described in more detail. Females of the constitution  $\begin{smallmatrix} S & + & + & + & Pfd \\ + & Cy & u & L & + \end{smallmatrix}$  were used because of the presence of the *Cy 2L* and *Cy 2R* inversions and their visible dominant markers. Each female and her mates were kept in separate bottles and transferred at intervals of 2 days through a total of five cultures, and the offspring from

the separate cultures were scored independently. In this and one other experiment the culture bottles were given code numbers, so that the experimenter who was looking for crossovers would not be influenced by knowledge of what kind of crossovers, if any, might already have been produced earlier by the same parent.

In the control series of eighteen females, less than 1% of crossovers were produced spontaneously (fig. 4, lower right

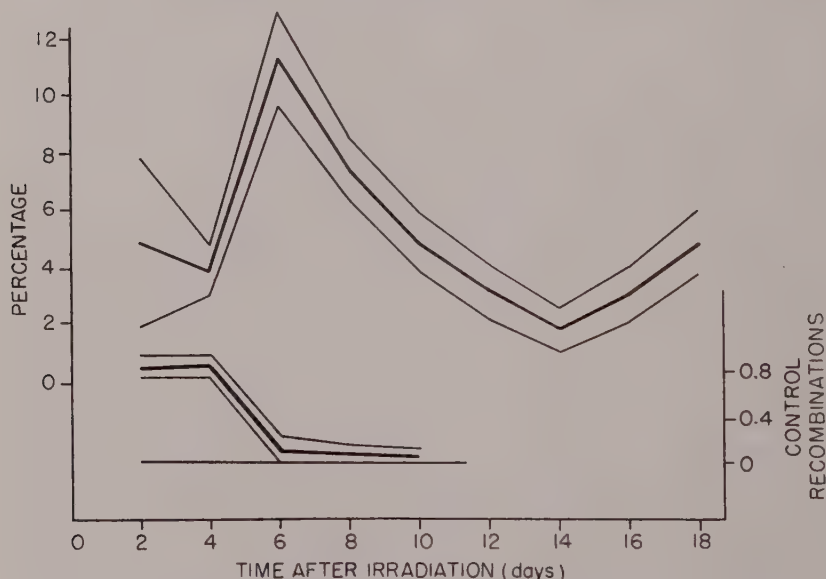


Fig. 4 Recombination values in consecutive testerosses of 28 *S Pfd/Cy lt L*<sup>4</sup> females X irradiated (2250 r) as virgin adults and of eighteen unirradiated controls.

scale). Surprisingly, these occurred in the first 4 days of egg laying and then almost disappeared from among the eggs of females 7–11 days old. This decrease parallels the known age effect of decreasing crossover values during the first week of life of young females as described by Bridges ('27) and Bergner ('28). The spontaneous crossovers not only fell off to zero during the same times as the age effect is usually expressed, but they were highly nonrandom. A

probability of only  $6 \times 10^{-15}$  has been computed for the chance distribution of these spontaneous recombinations to the individual families and regions (Whittinghill and Hinton, '50). These control data suggest that if spontaneous crossing over produced clusters of crossovers in inversion females, it may also be producing clustered recombinants from ordinary females. If so, this might be the explanation of the high variability from family to family described by Gowen in 1919.

The experiment in which X rays were used has been published by Hinton and Whittinghill ('50). Females X-rayed as young imagoes with 2250 r were transferred every 2 days for as long as they lived. At all times the recombination values in the X-rayed series were above those measured in the controls, and all of the increases were significant except in the first 2-day period. However, in spite of any increases in sampling error owing to mitotic multiplication of crossovers, there can be no question of some increase in recombinants following irradiation, since all of the eight crossover values beginning 4 days after X irradiation were above the five control values.

The peak of crossovers appearing in figure 4 is representative. It comes 5-7 days after irradiation, which is in general agreement with other induction experiments on females beginning with the early work of Plough ('17). The first interpretation suggested by this delay of 5 or more days before the laying of the eggs in which crossover values increased was that crossing over did not take place in the second maturation division but earlier, presumably in the first division. Soon it was recognized (Plough, '24) that the increase was initiated still earlier, i.e., in gonial cells. Such an interpretation is in keeping with the long-continued high crossover values found after the peak of recombination recovery as in figure 4. These same data may be analyzed to see whether they may readily have resulted from meiotic crossing over following X-ray treatment. If they did, they should be randomly distributed as to families. If the crossovers were not

random, then the earlier statements of Plough about a gonial effect would be confirmed.

The data of the same X-ray experiment are arranged to show family variability in table 5. The families of sizes from 556 to 199 are listed individually, but the smaller ones have been combined. A  $\chi^2$  test for homogeneity showed that the sums of the classes below 199 offspring did not differ significantly from the sums of the larger classes, yet a mere inspec-

TABLE 5

Testercross families from

X-irradiated (2250 r)

S

+

+

+

+

Pfd

+

+

Cy

lt

+

L<sup>A</sup>

females

		CROSSTOVERS					
		Region 1			Region 2		
FAMILY SIZE		S lt L <sup>A</sup>	Cy Pfd		S L <sup>A</sup>	Cy lt Pfd	
Individual families	556	13	7	←	0	1 double	0
	491	5	7		6		5
	477	1	1	→	12		5
	296	9	10	Both high	4		2
	288	5	2		5		2
	258	1	1	Both low	1		0
	201	3	0		2		2
	199	8	2	←	0		1
Σ 20 smallest families	953	17	14		16		5
Total	3719	62	44		46		22
Recombination		2.85 ± 0.28%			1.83 ± 0.22%		

tion of individual families reveals many examples of wide deviations from the over-all. For instance, the largest family had many crossovers, but all 20 were in region 1, between *Cy* and *lt*. The third family had almost as many, but mostly in the other region, 17:2. The fourth family, although much smaller than those mentioned, had a high number of crossovers in the first region and an average number in the second, or a 19 to 6 distribution between regions. The sixth family, which was of about the same size as the preceding, had un-

usually few crossovers in each region, a total of only three flies. The eighth family had a high value in the first region, but a low frequency in the second, in the ratio of 10:1. Such an uneven response would seem to rule out any interchromosomal influence of inversions, which might be present in some  $F_1$  flies but not in others. Lack of an even increase simultaneously in both adjacent regions indicates some gonial influence to explain the hitting or missing of a particular region in a particular parent.

The test for homogeneity between the group of smaller families and the combined larger ones deserves additional comment. A  $\chi^2$  test within the eight largest families shows a very significant lack of homogeneity. Yet when these were taken as a group, the group was not significantly different from the group of smaller families. Thus homogeneity has been artificially created by the lumping of data from the eight families, and that homogeneity was built up in respect to total crossovers, in respect to regions of exchange, and in respect to complementary classes. This illustration of crossovers averaging out may explain our past failure to recognize products of gonial crossing over as such in ordinary *Drosophila* females. Perhaps in other organisms gonial clusters average out and thus escape notice there also.

This X-ray experiment has been confirmed by another very similar study with  $\gamma$  rays performed at Oak Ridge National Laboratory by the author at the same time that Hinton was doing the X-ray experiments in Chapel Hill; the two were complementary in that the crossover phenotypes of one test were the noncrossovers of the other. This allowed an external check on viability differences, if any had been encountered in the gross data of either experiment. Table 6 is condensed from the published account of this  $\gamma$ -ray experiment (Whittinghill, '51).

The crossovers found after an exposure of pupae and young virgin adults to 4123 r of  $\gamma$  rays showed widely fluctuating frequencies of recombinants from family to family. Table 6 shows families individually down to size 180, together with



the contributions to  $\chi^2$ . There is less than a 2% chance that independently formed crossovers would vary this far or farther from expectation. The high  $\chi^2$  came chiefly from the second and third largest families. In addition to the computations, an excess number of six crossovers appeared in a small family of 45 flies, and the family of 596 with a reasonable total frequency of recombination had the unusual distribution of 16:4 between complementary crossover classes. Since neither of these last two facts has contributed as such to the

TABLE 6  
*Testcross families from eggs laid 5-21 days after  $\gamma$ -ray dose*

of 4123 r to						females	
FAMILY SIZE		CROSSEVERS				D <sup>2</sup> /E	
		S	Pfd	Cy	L <sup>A</sup>	Total	
Individual families	596	16:4 <sup>a</sup>				20	0.246
	554	11:13				24	3.409
	424	1:2				3	7.408
	186	2:2				4	0.457
	45 <sup>a</sup>	2:4				6 <sup>a</sup>	0.352
$\Sigma$ 7 smallest families	263	1:4				5	
Total	2068	33:29				62	11.872
3% Recombination		4 D.F.				$P < 0.02$	

<sup>a</sup> Other unusual distributions not influencing this  $\chi^2$  calculation.

high  $\chi^2$ , the likelihood of this distribution having resulted entirely from meiotic exchange is much lower than the 2% shown in table 6.

If none of the six irradiation experiments and one control series seem to be within limits of expectation based on independence of crossing over in meiosis, the question must be faced as to whether the nonrandomness results from (1) gonial crossing over or (2) gonial conditioning of chromosomes the descendants of which will cross over at the same region as each one undergoes meiosis. These are alternatives G and C of figure 1.

GONIAL CROSSING OVER VERSUS DELAYED CROSSING  
OVER OF DAMAGED CHROMOSOMES

Reference to figure 5 will show several features of gonial crossing over not imitated in meiosis. Gonial crossing over and case II segregation may initiate sectors of genotypically and phenotypically opposite cells. Then these sister cells homozygous for contrasting genes may multiply unequally for either of two reasons. Chance alone may determine that

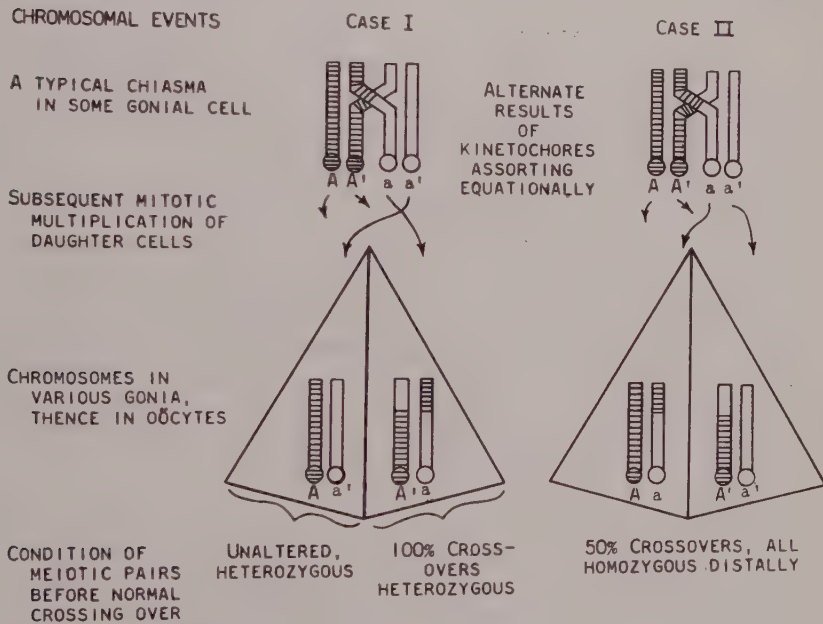


Fig. 5 Changes produced by gonial crossing over in the genotypes of certain germ cells before meiosis.

one of them produces more generations of gonial cells than the other; or genes affecting the viability of cells may deter the growth of one sector without in any way retarding the formation of germ cells by the other sector. Either agency could alter the proportions of complementary crossovers recovered in the next generation. Concurrently, noncrossovers would be affected to the extent that they come from the cell which had undergone gonial crossing over. Neither

of these effects would result from the weakening of a chromosome without gonial crossing over. Thus a distinction may be discerned between the two mechanisms of formation of the nonrandom crossovers already described. Complementary crossovers resulting entirely from conditioned meiotic (or from any meiotic) exchanges would have a random binomial distribution around a 1:1 ratio, or around some other ratio if larval viability differences favor one class. By contrast, the complementary products from gonial crossing over would have wider spreads around the obtained mean. The wider spread of gonially formed crossovers would go both ways if chance determined the more productive sector, but experiments in which lethal genes were used could be set up to favor deviations in one direction only.

The types of experiments which can be diagnostic are those in which deleterious or lethal genes enter the  $F_1$  generation from one parent, whereas a lethal-free chromosome comes in from the other  $P_1$ . Hence an isogenic wild stock was obtained by procedures which are standard for *Drosophila*, and it was mated in several experiments to stocks which contained one or more genes with recessive lethal action. In such experiments it may be predicted from figure 5 that the loss of one kind of crossover will be matched by the loss of a specifiable noncrossover. Hence a decision between gonial conditioning and gonial completion of crossing over may be made on the basis of inequalities between complementary crossovers and between complementary noncrossovers.

The first experiments were on flies which were heterozygous for the *Cy L<sup>4</sup>* balancer chromosome opposite an isogenic and therefore lethal-free second chromosome.  $F_1$  females were bred and transferred at 4-day intervals; their sibling  $F_1$  males were remated to suitable recessives every 4 days for five successive breeding periods. Various doses of X rays were given to the treated series before the young adults were testcrossed. The first cultures from each  $F_1$  were discarded, and classifications were made of the second, third, fourth, and fifth bottles. This embraced the period when the maximum

increases in recovered crossovers were to be expected. Figure 6 shows a comparison of changes in recombination values with changes in noncrossover distributions. These should be independent if all crossing over were delayed until meiosis. However, they would show an interaction following gonial crossing over and mitotic segregation as in case II. The data from cultures 2, 3, 4, and 5 are shown following each of the four total dosages. The larger diamonds signify the larger

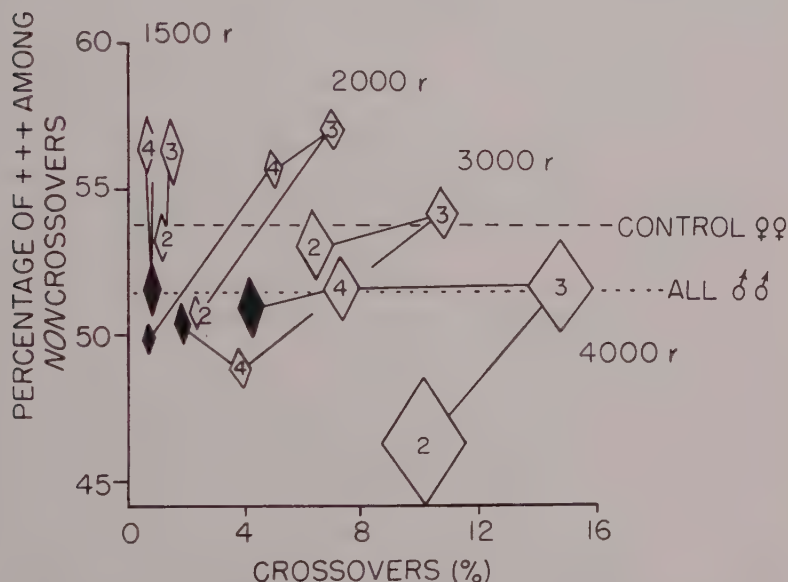


Fig. 6 Noncrossover segregation in relation to induced crossovers from X-irradiated *Cy lt L<sup>+</sup>/+* *Drosophila melanogaster*.

standard errors resulting from small families and larger percentages. The smaller diamonds represent smaller sampling errors. The solid diamonds denote the fifth cultures, by which time the four treatment series converged. In the 4000-r series it will be seen that substantial frequencies of crossovers were found in cultures 2 through 5 without any significant change from equality among the remaining noncrossovers. That this is not surprising will be explained later.

With intermediate dosages, correlated shifts of wild non-crossovers and both crossovers were noted. The 3000-r series data increased to peaks of crossovers and of wild noncrossovers in culture 3, decreased in both respects in culture 4, and finished in fair agreement with the other cultures 5. Similar correlation of total crossovers with wild noncrossovers appeared from the 2000-r treated females. Both were low in cultures 2 and 5, and both were high in cultures 3 and 4.

In the 1500-r series greater increases than expected were found among the noncrossovers. In view of the relatively smaller frequency of crossovers at this low dose, such a large increase in wild types from the control values up to 56.3 and 56.6% and then back to normal suggests some physiological effect, as opposed to the cytogenetic effects which were under deliberate consideration. No further explanation is available at this time.

The lack of a change among the noncrossovers after high dosage may be explained by remembering that a large number of new lethals are induced by a 4000-r treatment. Whenever these might be cell-lethals induced in the chromosome which was from the isogenic wild stock, that chromosome would lose its former viability advantage in sectors of the ovary becoming homozygous for it. Hence it might be eliminated, either by death or by retarded growth of its sector, about as readily as its homolog would be selected against. Thus it would be expected that a high proportion of the crossovers recovered from this radiation dosage would have come from crossovers segregating heterozygously as in case I of figure 5, and a low proportion from case II segregations. This, incidentally, might operate in any experiment designed to study a possible linear relation between ionization dose and recovered recombinations.

A final experiment was particularly conclusive as to the relation of recovered crossovers and extra noncrossovers of the wild class. The irradiated specimens were males from a different mutant stock, in which induction of crossovers was not as easy as in the previous experiments. Such differences



in susceptibility to crossover induction have been described before (Whittinghill, '37). Not only did hybrids from various stocks differ, but reciprocal matings of the same stocks revealed a maternal effect in their frequency of crossover production by high temperatures.

The genotype of the irradiated males is given in table 7. Inversions were present in both arms of the *Cy* chromosome, which were presumed to carry many other lethals besides the known lethal giant larvae<sub>3</sub>. It may be noted in the table that no appreciable rise in the crossover frequency was found before the seventh day and that a maximum of only 3% was

TABLE 7

*Testercross offspring of 19 lg<sup>l</sup> Cy cn<sup>2</sup> sp/isogenic wild males given 3000 r dose of X rays as young adults and mated to five successive groups of cn bw sp females*

DAY OF MATING	NONCROSSEOVERS		CROSSEOVERS		TOTAL	PERCENTAGE CROSSEOVERS	PERCENTAGE +++ AMONG NON- CROSSEOVERS
	<i>Cy cn sp</i>	+++	<i>Cy</i>	<i>cn sp</i>			
1	1477	1411	0	2	2,890	0.07	48.9 ± 0.93
4	596	568	1	1	1,166	0.17	48.7 ± 1.46
7	472	481	4	2	959	0.63	50.7 ± 1.62
10	1829	1833	12	40	3,714	1.40	50.1 ± 0.82
13	2743	2914	99	78	5,834	3.03	51.4 ± 0.65
Total	7117	7207	116	123	14,563	1.64	50.3 ± 0.41

realized in the offspring from zygotes formed 13-18 days after X irradiation. When crossovers were increasing, the wilds among the remainder were also increasing, from 48.9 to 51.4%. Although these two values alone are not significantly different, it may be shown that the last matings were significantly higher than the mean of the first four. Similarly, the entire body of data may be split between the second and third matings, or between the third and fourth, and each calculation shows that the later matings had significantly more wild types than the earlier matings. Such increases in two of the four kinds of offspring with increasing time after X irradiation were even more extreme in one of the nineteen families included in this table. Yet even without

this best example there was a fairly steady increase of cross-overs and of the noncrossover wilds in the remaining eighteen families. Hence, a special consideration of one family should be considered illustrative and without unwarranted bias.

Table 8 summarizes the offspring of the male which produced crossovers most extensively. The table shows that the wild-type noncrossovers had no original advantage over the *Cy cn sp* but that later they gained one. The gain accompanied the arrival of induced crossovers, and it was accentuated when only one class of crossovers, *Cy*, remained. In fact, the excess of wild noncrossovers ( $267 - 130 = 137$ ) about equalled the one class of crossovers, 132 *Cy*, in the final days of breeding.

TABLE 8

*Advent of inequality of complementary classes*

Broods of male number 213 following 3000 r of X rays administered at 35°C.

PERIODS OF TESTCROSSING	TOTAL OFFSPRING	NONCROSSEOVERS		CROSSEOVERS		PERCENTAGE + CHROMOSOME ARMS
		<i>Cy.cn sp</i>	+++	<i>Cy.++</i>	+.cn sp	
1st to 10th days	297	165	132	0	0	$44.4 \pm 2.89$
10th to 29th days	808	299	373	98	38	$54.6 \pm 1.75^a$
29th to 37th days	529	130	267	132	0	$62.9 \pm 2.10^a$
Totals	1634	594	772	230	38	3268 arms

<sup>a</sup> Wild types significantly above Mendelian expectation of 50%, and above actual mean of the whole experiment for 25 days,  $50.3 \pm 0.41\%$ .

The computation of percentage of wild chromosome arms includes all offspring this time and not merely the noncrossovers, thus indicating an increased transmission of wild-type genes and a decreased inheritance of lethals.

Reference to table 9 will show that the excess of wild-type offspring was closely matched by the number of induced cross-overs. There would have been no matching if crossing over had taken place in hundreds of primary spermatocytes. However, the hypothesis of gonial crossing over in one or two cells may be used to predict the number of wild-type flies starting from the other three classes. If 206 *Cy cn sp* flies were recovered in the testerosses, approximately another 206 wild flies would be expected from the unaltered part of the

testis. If 193 *Cy* crossovers were recovered following the maturation divisions, a sample of the same size would be expected containing whatever chromosome had been paired with the *Cy* up until meiosis. Such another chromosome would certainly not be *cn sp*, which is too sparsely represented in the data, nor could it be the *Cy cn sp* noncrossover for the same reason and because of suspicion of the cell lethality of the homozygous inversions carrying *cn*<sup>2</sup> and *sp*; hence the contribution of about 193 more wilds to that noncrossover class is predicted. The sum of these two predictions for wilds is now close enough to the actual figure to be within

TABLE 9

*Prediction of the numbers of wild-type offspring on the basis of the other classes and twin-spot mosaicism through spermatogonial crossing over. Data from all test crosses of male 213 between 18 and 37 days after irradiation. (Total offspring, 825.)*

	NONCROSSEOVERS		CROSSEOVERS	
	<i>Cy cn sp</i>	+++	<i>Cy ++</i>	+ <i>cn sp</i>
Types obtained	206	413	193	13
from normal sector	206	206		
from <i>Cy</i> sector		193	193	
from <i>cn sp</i> sector		13		13
Wilds predicted		412		

the range of normal sampling. If in addition the 13 *cn sp* are considered, they may be paired with any other class without spoiling the prediction. Ideally, they may be supposed to have been paired with the isogenic wild chromosome, and so some 13 more flies of the wild phenotype may be predicted. The sum of these estimates is 412 flies; the number obtained was 413. Thus it would seem that in this family segregation was of the type by which twin spots of partially homozygous tissues were formed in the testis. It further appears that wild-type chromosomes multiplied readily but that the lethal-bearing *lgl Cy cn*<sup>2</sup> *sp* chromosome was often killed off by distal homozygosis before gametogenesis.

The two hypotheses may be compared in a detailed examination of the entire breeding period of this male (fig. 7). The expectations are (1) equality of noncrossovers following meiotic crossing over, and (2) equality of wild noncrossovers with the sum of all remaining classes following gonial crossing over and case II segregation. The differences are expressed in numbers of testcross offspring. Standard errors above and below the obtained difference on the meiotic hy-

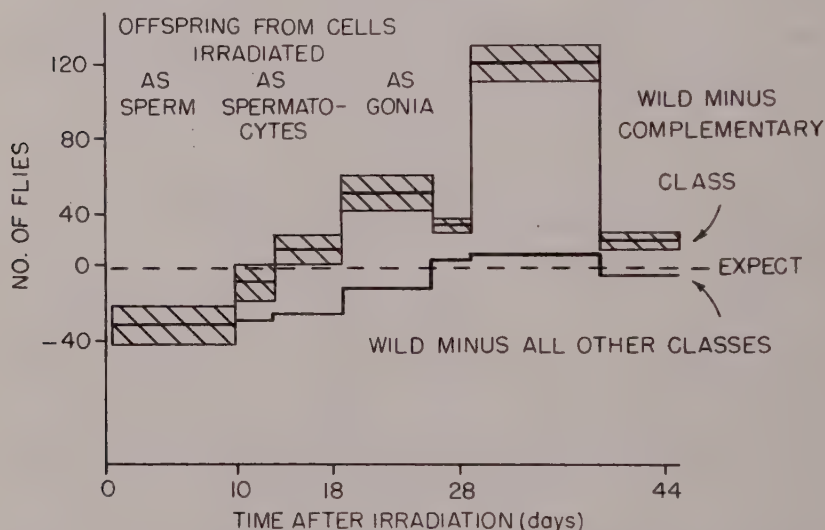


Fig. 7 Differences between wild noncrossovers and other testcross offspring of *lgl Cy cn<sup>2</sup> sp/+* male number 213 from successive matings and transfers.

pothesis are drawn in. It will be seen that from the eighteenth day onward through four different periods of breeding the wild class was significantly more numerous than its complementary noncrossover class. The excesses were 45, 25, 121, and 16 flies. However, these flies, which are too numerous on the hypothesis of meiotic crossing over, are fairly well matched by crossover classes. A regrouping of the data on the assumption of a gonial origin for all crossovers would compare all wild types with all crossovers plus the other noncrossover class. The differences within this comparison are slightly

above or slightly below the zero line. Their standard errors have been omitted for clarity, but they are almost as large or are larger than the actual differences. Because of this agreement with the second hypothesis it may be concluded that the gametes used after the eighteenth day contained descendants of chromosomes which were surely in gonial cells at the time of irradiation. The stage of the germ line being sampled is indicated crudely in figure 7; evidence from other experiments would indicate that cells irradiated as spermatogonia have given rise to functional sperm by the tenth day.

#### DISCUSSION

Both  $\gamma$  and X rays affect crossing over in both sexes of *Drosophila*, giving increases in the centromere regions of females and inducing crossing over chiefly in the same regions in males. The work with induced crossing over has not yet gone far enough to detect any differences, if they be slight ones, between  $\gamma$ - and X-ray treatments or between ionizing radiations and thermal effects. Much of the difficulty of making the comparison, and in attempting studies of the relation of dosage to effect, lies in lack of knowledge of how to deal with the variable percentages of recombination caused by uneven multiplication of one or both crossover chromosomes after the actual event of crossing over.

It cannot be decided whether the mode of induction in normal females is the same as in normal *Drosophila* males, but the rare crossovers from female inversion heterozygotes suggest that the mechanism may be the same. Knowledge of high variability in normal females and demonstration of a nonrandom age effect in the *Cy* inversion females confirm the suggestion that crossing over in the germ line is not limited to the first meiotic division. Inspection of the male data, and of certain experiments with females point clearly to the initiation and completion of crossing over in spermatogonial and even in oögonial cells. Because of this completion of the exchange, it is possible to demonstrate selection against lethals before germ cell formation, so that gametic



ratios for the favored class rise significantly above the Mendelian expectation of 50%.

The *Drosophila* work has several implications for other organisms. If the action of ionizing radiation is entirely gonial, alterations in crossover values may be impossible in some other forms, such as *Neurospora*, in which no diploid nuclei precede the primary gametocyte stage. Where somatic twin spots are known, such as in maize, induced recombinations might be possible in the clustered manner of *Drosophila*. If, on the other hand, some of the crossing over induced in *Drosophila* is meiotic in origin, then *Neurospora* and a wider variety of plants and animals might respond to the action of inducing agents.

The fact that lethal genes may be selected against before gametogenesis may have had evolutionary importance to *Drosophila*. With such selection in operation, the species may have been able to tolerate a slightly higher mutation rate than organisms in which partial homozygosis in the germ line did not intervene before gamete formation. It has long been suggested that the mutation rate for *Drosophila* may be higher than average. If the contribution of gonial crossing over to evolution in the genus *Drosophila* should be established as an important one, in the elimination of any substantial number of lethal alleles, the use of radiation for this kind of improvement of some other organisms of economic importance might be indicated.

#### SUMMARY

1. X and  $\gamma$  rays are able to induce crossing over where it does not occur spontaneously, in *Drosophila* males, and to increase crossover production by *Drosophila* females of widely different constitutions. In both respects these agents parallel the effects of high temperature treatments.

2. The inductions in males and the increases in females show similarities as to time of appearance of crossovers, region of the chromosome usually affected, and lack of

randomness. These induced recombination offspring are nonrandom as to families, nonrandom as to regions within families, and often nonrandom as to complementary classes within a region.

3. Spontaneous crossovers occurring early from *Cy L<sup>4</sup>* females also show nonrandom distributions and therefore indicate a possible gonial influence in structurally homozygous females.

4. Correlated recoveries of one crossover and of a lethal-free noncrossover chromosome establish the fact that the process of crossing over may be stimulated and completed in a few cells long before meiosis.

5. Implications of gonial crossing over for *Drosophila* and possibly for other organisms have been raised.

#### DISCUSSION

MEYER: We recently found ultraviolet to be capable of inducing crossing over in the male germ line. The preferred regions for this type of crossing over likewise seem to be those near the centromere. Since we treat the pole cells of the embryos, we recover clusters of identical crossover recombinants among the offspring, in most cases, and conclude that crossing over took place in immature germ cells long before meiosis.

WHITTINGHILL: There might also be some delayed crossing over at meiosis, and it is difficult, as I have shown, to distinguish it from early completed crossing over.

ATWOOD: Do you take for granted that these exchanges are exactly homologous, or do you have some actual evidence?

WHITTINGHILL: In some experiments (Whittinghill, '37) I have tested the viability of these by getting the crossover strand in homozygous condition without further crossing over. In the vast majority of these tests the homozygotes survived; and therefore we have concluded that these were typical crossover exchanges.

ATWOOD: The distribution of exchange regions looks much more like a break-frequency map than a linkage map. The

regional distribution when the crossing over is increased by *Minutes* is not the same as when it is increased by X rays. These exchanges could perhaps be thought of as translocations between homologous arms, with breaks usually close together in the heterochromatic region. With this kind of translocation, no single gamete can simultaneously carry both of the reciprocal exchange products. This means that the cases where the breaks are far enough apart to give inviable deficiencies or duplications are effectively screened out by the time the exchange individuals are recovered. Thus the criterion of fertility of the supposed crossover flies is not sufficient to rule out a translocation mechanism for the exchanges. Normal salivaries might be a good criterion, but a large series would have to be examined because only those duplications and deficiencies which are in euchromatin would be recognized.

WHITTINGHILL: That would be true for the majority of crossovers which have been formed in large heterochromatic regions. But a substantial number of crossovers formed outside the centromere region have likewise been found to be viable when homozygous, yet the possibility remains that we may be dealing with two or more kinds of exchanges at various times after irradiating.

PARKER: I have irradiated *Drosophila* females (heterozygous for "3ple") shortly after emergence. All females were mated individually, and transferred to new culture vials daily, with egg counts being made. There were significant increases in crossing over on the first through the fourth day of egg laying, the increases being found in the most distal region (*roughoid* to *hairy*) on the first 2 days, in an intermediate region (*hairy* to *scarlet*) on the third day, and near the centromere (*scarlet* to *peach*) on the fourth day. Approximately half of the increase observed on the first day is in double crossovers, with both regions of exchange being in the same chromosome arm; increases on other days are largely due to single crossovers. From the sixth through the twelfth day, increases are found in one or more regions,

all but the most distal region (*roughoid-hairy*) being represented.

On the ninth, tenth, eleventh, and thirteenth days there is a significant decrease in crossing over between *roughoid* and *hairy*. The observed reduction is about three times as great as that expected if the reduction is due to homozygosis at the *roughoid* and *hairy* loci resulting from gonial crossing over induced medial to these loci.

The frequency of crossing over between *scarlet* and *peach* on the first day is about 8%, the value dropping rapidly to about 4% by the third day. On every day the treated series shows higher value than the controls, although the differences are not significant until the fourth day, when approximately the 135th to 180th eggs are laid.

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# VARIATION IN GENETIC RECOMBINATION IN NATURAL POPULATIONS

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THREE FIGURES

Recent years have witnessed an increasingly wide acceptance of the view that important evolutionary changes may be effected through recombination of polygenes. The concept of the polygene as a gene with small individual effect was introduced by Mather ('43) who developed the theory of evolutionary adjustment through intensive and extensive recombination of many such genes. Although the original scheme of Mather has undergone some refinements (e.g., Lerner, '50), the concept remains as one of the most important working hypotheses of population genetics. A side effect of this emphasis has been to bring into clearer focus the basic biological importance of genetic recombination through crossing over.

Crossing over has generally been studied at the level of the individual. Almost nothing is known of patterns of recombination as they exist in natural populations, yet such information constitutes the basic knowledge in any appraisal of the evolutionary potential of a species or population. The amount of genetic recombination may vary enormously among species. Some organisms, for example, have cast off all means of recombination; in such forms, which are essentially reduced to asexual reproduction, genetic diversity is fixed. Even when a mutation occurs, it finds itself in a rigid genetic milieu. Usually, however, such complete fixation is avoided and natural selection appears to favor a compromise such that genetic fixation is permitted to involve only a part of the

genome, while in other parts free recombination of genes may continue to occur. Retention of a high degree of recombination through crossing over imparts evolutionary flexibility to a species or population. This paper is concerned with the preparation of an index of crossing over for *Drosophila* populations which will reflect this potentiality for change.

The genus *Drosophila*, along with the rest of the higher Diptera, has suffered marked reduction in intensity of recombination through the elimination of crossing over in males. In most species of the genus, furthermore, the accumulation of chromosome inversions in coadapted systems results in still further restrictions on crossing over by interfering with free recombination in females. The net result of this situation is that populations of such species are made up of constellations of specific structural karyotypes. Karyotype heterozygotes, as compared with complete structural homozygotes, are characterized by a relatively reduced crossing over in chromosome sections which are relatively inverted. Although some compensatory increase of crossing over occurs in other chromosome sections (Carson, '53), free recombination in complex structural heterozygotes is confined to a relatively few such sections.

In the analysis which follows, different populations of *Drosophila robusta* Sturtevant will be described in terms of the relative amount of free recombination which is permitted by the inter- and intrachromosomal effects of the various naturally occurring inversions on crossing over. It will be shown that marginal populations are the most flexible in the immediate microevolutionary sense because they permit a relatively greater amount of free recombination.

#### MATERIAL AND METHODS

The data on which this study is based are those given by Carson and Stalker ('47, '49), Stalker and Carson ('48), and Levitan ('51a, b). In addition, Dr. Max Levitan has generously supplied some additional data, and some unpublished data of the writer have been included. The data have been re-sorted

and selected in such a way that "egg samples" of collections made prior to August 1 were used almost exclusively. Each egg sample consists of the complete structural karyotype observed in a single salivary gland smear of an  $F_1$  female larva produced by each wild female. No data from males are included.

The polytene chromosome complement of *D. robusta* consists of six long euchromatic arms and a small dot segment. These strands correspond to the left and right arms respectively of the three pairs of V-shaped chromosomes (X, 2, and 3) and the dot pair (4) of the metaphase. With a method previously described (Carson, '53), tracings of these strands permit the division of the entire euchromatic genome of this species into sections, the length of which may be expressed in terms of its percentage of the total euchromatin. Idiograms of *D. robusta* will be found in Carson and Stalker ('47) and in Carson ('53), the latter being the more accurate. Table 1 shows the relative length of each euchromatic section expressed in terms of the percentage of the total euchromatic chromosome length it occupies.

The effect of an inversion on crossing over depends on (1) its length, (2) its position in the chromosome arm relative to the position of the centromere, (3) its precise karyotypic context with respect to other inversions in the same and in other chromosomes, and (4) its frequency in the population. Sufficient general information on the effects of inversions on crossing over (Sturtevant and Beadle, '36; Schultz and Redfield, '51) and specific information for *Drosophila robusta* (Carson, '53) are available to permit quantitative measurement of these effects for individual females. Such a measurement may be referred to as an "index of free crossing over." The index is an expression of the percentage of the total haploid euchromatic chromosome length of the chromosomes of an individual in which free crossing over may occur, and is prepared by measuring the euchromatic length of the entire polytene chromosome complement and equating this to 100. The length of the chromosome segments in which crossing

over is blocked is then measured in percentage of the total. The total blocked length is subtracted from 100, leaving the percentage of the total in which free crossing over occurs. A chromosome segment is arbitrarily considered to be "blocked"

TABLE 1  
*Relative lengths of the euchromatic sections of the salivary gland  
chromosomes of Drosophila robusta*

CHROMOSOME ARM		REGION	PERCENTAGE OF TOTAL EUCHROMATIN
Chromosome X	Left	1	1.1
		2	3.3
		3-6	8.4
		7	1.9
		8	3.8
	Total		18.5
	Right	9	1.9
		10	3.3
		11	6.5
		12-13	4.5
		14	2.1
		15	1.2
	Total		19.5
	Total	1-15	38.0
Chromosome 2	Left	16	5.7
		17	0.8
		18	5.0
		19-20	5.0
		21-23	6.8
	Total		23.3
	Right	25	8.9
		26-27	9.4
	Total		18.3
	Total	16-27	41.6
Chromosome 3	Left	28	2.3
		29	9.1
	Total		11.4
	Right	30	1.7
		31	3.7
		32	1.9
		33	0.7
	Total		8.0
	Total	28-33	19.4
Chromosome 4		34	1.0

if crossing over occurs there in the formation of no more than 1% of the gametes. In most cases the amount of crossing over is very much less than this. A male *Drosophila*, in which no crossing over occurs at all, would thus have an index of free crossing over of 0; a female having a structurally homozygous karyotype would have an index of 100.

In the preparation of the index, all of the chromosome section located within a segment heterozygous for an inversion is considered to be blocked to crossing over. Although it is known that inversions suppress crossing over in sections of chromosome adjacent to them, this suppression has not been taken into account, since there is little basis as yet for accurately estimating it. Thus even a small chromosome section distal to an inversion is assumed to have free crossing over. In complex multiple inversion heterozygotes, especially those involving an inversion in each arm of a V-shaped chromosome, it is known that in the majority of karyotypes crossing over is suppressed in the entire region between the inversions. This region may thus be added to the blocked areas in such heterozygotes. In a small number of karyotypes, interchromosomal boosting effects release certain regions from their intrachromosomal suppressions. Such effects have been included in the index.

The structural chromosomal conditions encountered relatively commonly in wild populations of *Drosophila robusta* are listed in table 2. The symbols used follow the system described by Carson ('53). For example, an X-chromosome pair heterozygous for XL/XL-1 in the left arm but homozygous for the standard sequence XR in the right arm is written, X: S S/1 S. The S in each case stands for the "standard" sequence, the left arm being represented by the symbol to the left in each case.

Table 2 also gives the chromosome regions blocked to crossing over by each inversion or combination of inversions and the percentage of the total euchromatin covered by them. The total blockage for a particular female is thus obtained



TABLE 2

*Blockage of crossing over by naturally occurring structural karyotypes of Drosophila robusta*

STRUCTURAL CONDITIONS IN CHROMOSOMES	CROSSING OVER BLOCKED IN REGIONS:	PERCENTAGE OF TOTAL EUCHROMATIN BLOCKED TO CROSSING OVER
CHROMOSOME X		
<u>1</u> - / 2 -	2-7	13.6
<u>S</u> - / 1 -	3-7	10.3
<u>S</u> - / 2 -	2-6	11.7
- <u>S</u> / - 1; - <u>2</u> / - 1; - <u>3</u> / - 1	10-15	17.6
- <u>2</u> / - 3; - <u>S</u> / - 2	11-14	13.1
- <u>S</u> / - 3	12-13	4.9
<u>S</u> 1 / 1 2; <u>S</u> <u>S</u> / 1 1 <sup>a</sup>	3-15	33.6
1 <u>S</u> / 2 2; <u>S</u> <u>S</u> / 2 2	2-14	35.7
<u>S</u> <u>S</u> / 1 2	3-14	32.5
1 1 / 2 2; <u>S</u> 1 / 2 2	2-15	36.9
CHROMOSOME 2		
<u>S</u> - / 1 -	18-20	10.0
<u>S</u> - / 2 -; 1 - / 2 -; 3 - / 2 -	17-23	17.6
<u>S</u> - / 3 -	19-23	11.8
1 - / 3 -	18-23	16.8
- <u>S</u> / - 1	26-27	9.4
<u>S</u> <u>S</u> / 1 1	{ 18-27	35.1
	{ 18-20; 26-27 <sup>b</sup>	19.4
<u>S</u> <u>S</u> / 2 1; 1 <u>S</u> / 2 1; 3 <u>S</u> / 2 1	{ 17-27	35.9
	{ 17-23; 26-27 <sup>b</sup>	27.0
<u>S</u> <u>S</u> / 3 1	{ 19-27	30.1
	{ 19-23; 26-27 <sup>b</sup>	21.2
1 <u>S</u> / 3 1	{ 18-27	35.1
	{ 18-23; 26-27 <sup>b</sup>	26.2
CHROMOSOME 3		
<u>S</u> <u>S</u> / <u>S</u> 1	31-32	5.6
<u>S</u> <u>S</u> / L-R	29-31	14.5

<sup>a</sup> In these and a number of other double-inversion configurations, alternate linkage types are encountered, e.g., in this case S 2 / 1 1 and S 1 / 1 S. Two such alternate arrangements have similar structural effects and have not been distinguished in this paper.

<sup>b</sup> When chromosome X is also doubly heterozygous, blockage is confined to these regions.

by summing the percentages of the individual euchromatic segments blocked. Thus a female of the karyotype

$$\begin{array}{ccc} \text{S} & \text{X} & \text{S} \\ \hline 1 & & 1 \end{array} \quad \begin{array}{ccc} \text{S} & 2 & 1 \\ \hline \text{S} & & \text{S} \end{array} \quad \begin{array}{ccc} \text{S} & 3 & \text{S} \\ \hline \text{S} & & 1 \end{array}$$

would have a blockage of 33.6 (in X) plus 9.4 (in 2R) plus 5.6 (in 3R) or a total of 48.6. When the total figure is subtracted from 100, the remainder (51.4) is the percentage of euchromatin in which free crossing over occurs, or the index of free crossing over. Note that the blockage in the X chromosome in this case is more than the sum of the blockage by each of the inversions XL-1 and XR-1 separately, owing to the suppression of crossing over in the central region between them (Carson, '53).

When inversion heterozygosity reaches a certain level, the suppressive effects of the inversions on the sections between them, especially in chromosome 2, are overcome by the interchromosomal boosting effects of the inversions on crossing over. Thus, in the quadruple heterozygote

$$\begin{array}{ccc} \text{S} & \text{X} & 1 \\ \hline 2 & & 2 \end{array} \quad \begin{array}{ccc} \text{S} & 2 & \text{S} \\ \hline 1 & & 1 \end{array} \quad \begin{array}{ccc} \text{S} & 3 & \text{S} \\ \hline \text{S} & & 1 \end{array},$$

crossovers are forced out of regions 20-25 of chromosome 2 and the blockage in this chromosome is reduced accordingly (see values marked by superscript <sup>b</sup> in table 2). The blockage for this female would be: 36.9 (in X) plus 19.4 (in 2) plus 5.6 (in 3R) or a total of 61.9. Such karyotypes are actually few in number; crossing over in the central region of the X chromosome between two inversions occurs only very rarely, under any conditions.

## RESULTS

From the blockage measures given in table 2, an index of free crossing over was assigned to the karyotype of each of 614 egg samples of female larvae of *Drosophila robusta*. The wild flies came from nine geographical areas within the range of the species. Frequency distribution histograms of these indices, grouped in classes of five, are given in figure 1 for

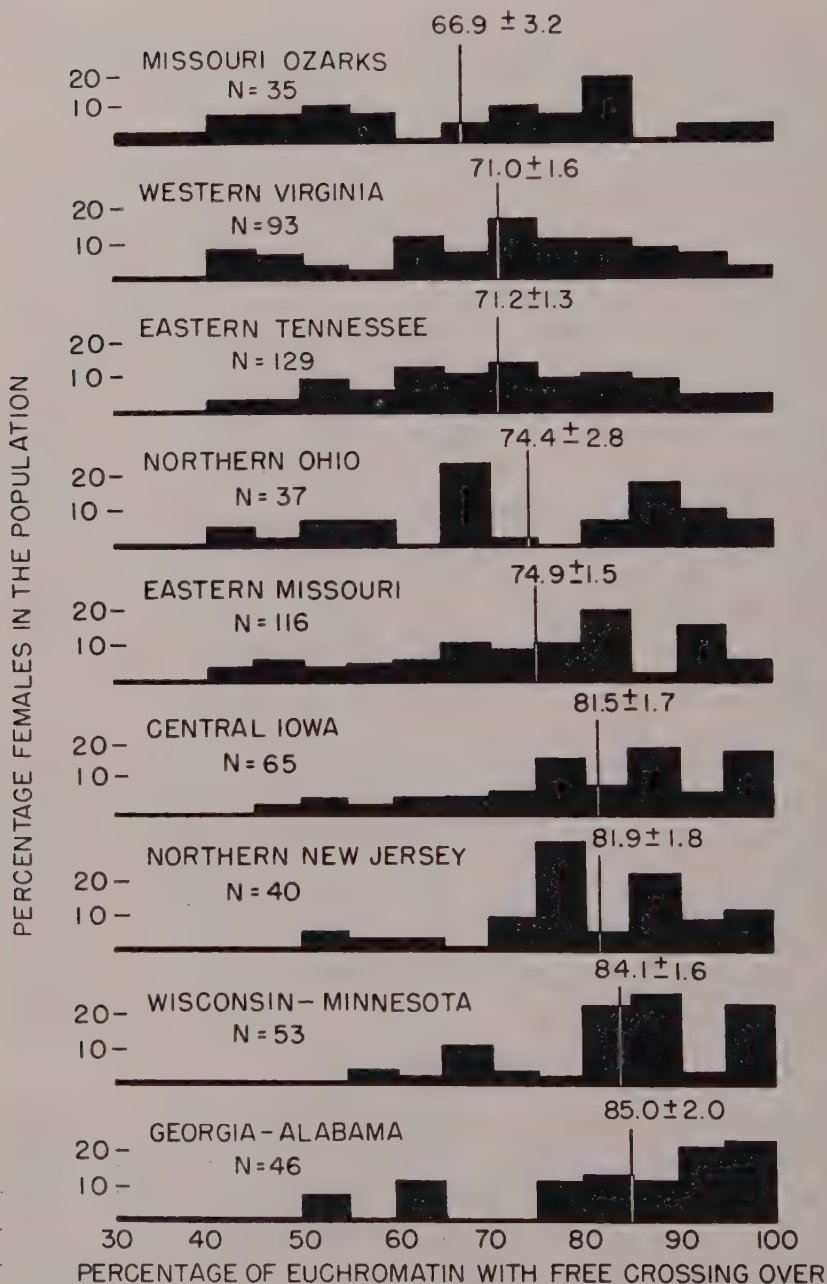


Fig. 1 Patterns of genetic recombination in females from nine different populations of *Drosophila robusta*.

each of these populations. The sample size is given in each case and the population means, with standard errors, are shown. Reference to figures 1 and 2 will show that in general the populations from the center of the distribution area — i.e., Missouri Ozarks, Western Virginia, Eastern Tennessee (open circles on figure 2) — show relatively low indices of free crossing over. Populations of intermediate geographical position



Fig. 2 Approximate geographical distribution of *Drosophila robusta* (hatched) and location of the nine populations considered in this study. Central populations are represented by open circles, intermediate by solid circles, and marginal populations by solid squares.

between these and the marginal populations are less blocked, whereas marginal populations — i.e., Wisconsin-Minnesota and Georgia-Alabama (solid squares on figure 2) have the highest mean percentages of free recombination. This general tendency may be observed by rough inspection of the histograms in figure 1.

Although the values appear to form a geographical gradient, or cline, certain discontinuities are apparent. These are not well demonstrated by the means, which are affected by the fact that some of the curves are skewed. Therefore a simple  $\chi^2$  test for differences between populations was applied to the number of individuals of each population which fall above and below the arbitrary point of 80% of free euchromatin (see table 3). On this basis, the populations fall into three

TABLE 3

*Differences in genetic recombination in various populations of Drosophila robusta*

SOURCE OF POPULATION	NO. OF FEMALES	MEAN PERCENTAGE EUCHROMATIN WITH FREE CROSSING OVER	PERCENTAGE OF FEMALES HAVING 80-100% FREE CROSSING OVER
CENTRAL POPULATIONS			
Missouri Ozarks	35	66.9 $\pm$ 3.2	31.4
Western Virginia	93	71.0 $\pm$ 1.6	30.1
Eastern Tennessee	129	71.2 $\pm$ 1.3	29.5
Total	257	70.5 $\pm$ 1.0	30.0
INTERMEDIATE POPULATIONS			
Northern Ohio	37	74.4 $\pm$ 2.8	45.7
Eastern Missouri	116	74.9 $\pm$ 1.5	46.0
Central Iowa	65	81.5 $\pm$ 1.7	55.4
Northern New Jersey	40	81.9 $\pm$ 1.8	47.5
Total	268	77.6 $\pm$ 1.0	48.5
MARGINAL POPULATIONS			
Wisconsin-Minnesota	53	84.1 $\pm$ 1.6	77.4
Georgia-Alabama	46	85.0 $\pm$ 2.0	71.7
Total	99	84.5 $\pm$ 1.3	74.7

homogeneous groups, which differ significantly from one another at or below  $p$  values of 0.05. These groups are given in table 3 and figure 3. They consist of the three most central populations (1, 2, and 3), four intermediate (4, 5, 6, and 7), and two marginal (8 and 9).

#### DISCUSSION

The data presented show clearly that as one approaches the marginal areas occupied by the species, the amount of free crossing over increases. This is, of course, a reflection of



the finding that marginal populations are relatively homozygous for gene arrangement whereas central populations are relatively heterozygous. This same tendency may be observed in the clines of gametic frequencies for certain individual

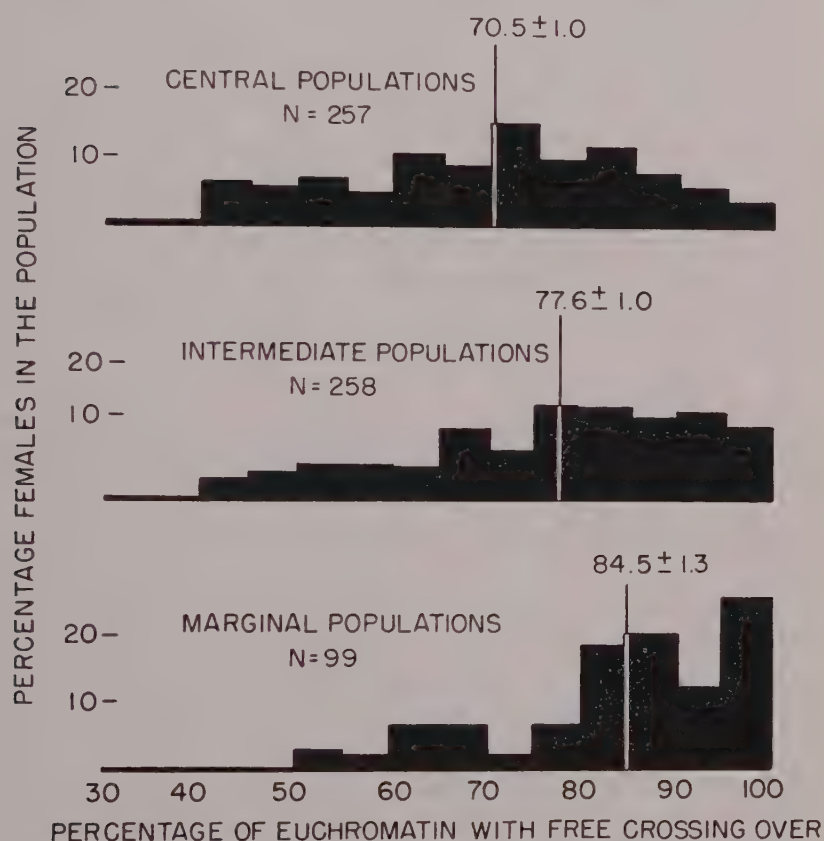


Fig. 3 Differences in free recombination in central, intermediate and marginal populations of *Drosophila robusta*.

gene arrangements. In general, the frequencies appear to be approaching 0 or 100% at the margin of the area occupied by the species (Carson and Stalker, '47; Stalker and Carson, '48). Similar relations obtain in *D. persimilis* and apparently to a lesser extent in *D. pseudoobscura* (Dobzhansky, '44, '48).

The histograms shown in figures 1 and 3 constitute a more direct measure of the phenomenon of marginal homozygosity without the complicating factor of qualitative differences between different marginal populations.

It is striking that two populations which are geographically and ecologically so different as Wisconsin-Minnesota and Georgia-Alabama should have frequency distributions and means which are so similar (fig. 1). This is so despite the positions of these populations at opposite ends of the north-south clines which have been demonstrated for this species. These populations are different both chromosomally and morphologically (Carson and Stalker, '47; Stalker and Carson, '47) and these differences could be easily used to erect subspecies if desired. The similar indices of free recombination of such different populations therefore require an explanation.

Da Cunha and Dobzhansky ('54) have recently added further data which support the hypothesis made earlier (da Cunha *et al.*, '50) that the amount of adaptive polymorphism in a given population is directly proportional to the number of adaptive niches occupied and exploited by the members of that population. They have thus shown that marginal populations of *D. willistoni* tend to have relatively less chromosomal polymorphism than populations inhabiting central or ecologically more diverse areas. The data available for *D. robusta* support this hypothesis. The indices presented in this paper are a somewhat more sensitive measure of structural population homozygosity than the average number of heterozygous inversions per individual which has been used by da Cunha *et al.* ('50) for *D. willistoni*.

*Drosophila robusta* is geographically and ecologically in a marginal situation in both Georgia and Wisconsin. Very few specimens of the species have been captured in Florida and the combination of the disappearance of the American elm and the ecological ascendancy of the pine forest appear to be the leading factors in determining its southern limits. In the

north-central part of its range, the species has not been caught north of Itasca Lake, Minnesota. Although suitable breeding sites on elms are available farther north, the coniferous forest is nearby and the species appears to be replaced in the North by its close relative *D. colorata*. It may be concluded that the extreme northern and extreme southern populations of *D. robusta* are similar because both are marginal and both are able to exploit only a limited number of ecological niches.

From the point of view of population genetics, the observed distributions of inversions in this species appear to reflect two fundamental phenomena. The first of these is that, in the central or ecologically complex portions of a species range, accumulation of chromosomal polymorphism results in considerable specialization. Adaptive gene complexes are held together and recombination is lessened. Coadaptation of chromosomes, however, is a plastic system because it is based on the superiority of the heterozygote. If the adaptive value of the heterozygote should in the course of time become lower than that of one of the homozygotes, one of the gene arrangements would be eliminated from the population. The result would thus be a return to homozygosity for this chromosome section, and free recombination in this part of the genome would be restored.

The second phenomenon concerns the converse tendency, that is, for the marginal populations to have a high potential for recombination. In contrast to central populations, marginal populations may be considered to be less specialized and what variability they have would be more easily and continually available through polygenic recombination. Marginal populations should thus be genetically more flexible in terms of immediate evolutionary adjustment to new conditions than central ones. Nevertheless, the ability of marginal populations to make use of this potential depends on the store of genetic variability available to recombination, the degree of inbreeding, and population size. Further information on these factors is a necessary prerequisite to further analysis.

## SUMMARY

The complete structural karyotypes of 614  $F_1$  female larvae from wild females of *D. robusta* were recorded. For each karyotype the percentage of the total chromosomal material which is available to crossing over has been calculated. This is referred to as an index of free crossing over.

Frequency distributions and population means were prepared for the indices of free crossing over for each of nine geographical areas. Centrally located populations have a relatively low percentage of free recombination; populations from the margin of distribution have conversely a relatively high amount of free recombination. Populations of intermediate position have intermediate values. These correlations are interpreted to mean that central populations are genetically more specialized, many genes being effectively tied up in nonrecombining coadapted groups. Marginal populations, on the other hand, tend to be structurally homozygous and have a greater free recombination. Marginal populations are thus genetically more flexible in the sense of capacity for immediate evolutionary adjustment to new conditions. This is possible because of their greater potentiality for adjustment through extensive recombination of polygenes.

## ACKNOWLEDGMENTS

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## DISCUSSION

BAKER: I was interested in the case where inversions in both arms of the X increased the frequency of recombination



in a centromere region, which I guess was heterochromatic. Do you have any evidence of an increased frequency of crossing over by the interchromosomal effect actually within heterochromatic regions of the chromosome?

CARSON: The region in which crossing over is being detected in the second chromosome consists both of two heterochromatic segments adjacent to the centromere and two quite long euchromatic segments as well. It has not been possible to tell precisely where the increased crossing over is occurring.

BEADLE: Might you not expect that simple size of breeding populations would lead to this same result, that at the periphery you get small surviving breeding populations that would lead to homozygosity?

CARSON: Yes, I think this is certainly a valid hypothesis. However, the gradual clinal changes which have been shown in this species indicate that something more than population size is playing a role at the margin because of the gradual approach to the low frequencies of inversions. If the marginal populations were isolated, this might be a different matter, but they appear to be in contact.

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## EVALUATION OF RECOMBINATION THEORY

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The three of us who were asked to speak at this evaluation session have discussed the matter and agreed that we are at somewhat of a disadvantage since none of us has had too much experience in bacterial genetics, phage, or biochemical material. For my own part, I can only say that I have learned a good deal, but would want much more time to digest the material before venturing to make more than a few passing comments

I should, however, like to say a few words about the scheme that Dr. Lederberg has called "copying choice." This general type of scheme for the production of recombinations seems to me, as far as I can judge, to have very great advantages in the interpretation of the recombinations in phage. But at least in its original form, I think it will involve very serious difficulties and will require some modifications to account for what goes on in chromosomes. The chief difficulty is that there is no provision in this scheme for the simultaneous production of the contrary crossover types. As you know, when an exchange occurs in a pair of chromosomes, the two contrary genetic crossovers are produced simultaneously.

It is quite clear that this is the regular result in chromosomes but is not the rule in phage recombinations. The two types of chromosomes are recovered together, but the contrary phage recombination types are not necessarily recovered from single infected bacteria.

This makes me wonder whether it is too hopeful to try to explain what goes on in the phage recombinations and in chromosome recombinations on the same basis. It should be remembered that in these two situations, objects of very dif-

ferent sizes are considered. A chromosome is, of course, a good many orders of magnitude larger than a phage particle; and there are some differences in the phenomena that are observed, of which the above is one of the more striking.

Of course, we should all like to see a general scheme that would apply to both situations, but it seems to me we had better be prepared to find that the mechanism is quite different in the two cases.

In passing, I should like to make a comment in connection with the point which Dr. Crick has made to the effect that one of the striking things about chromosomes is that they do not come in branched form. I think I am one of the few people who have ever suggested the existence of a branched chromosome. I should like here to retract that suggestion. The case which seemed to me to be conclusive certainly has a different explanation, as the analysis of the salivary gland chromosomes shows.

Another point which I wish to discuss a little is one which was mentioned by Dr. Lederberg, namely, the examples which occur in the fourth chromosome of *Drosophila*, of extremely short double crossovers, where the two exchanges are very close to each other.

I have been much puzzled by these data, and have tried to find some way to account for them. The only suggestion I can make as to what may be going on is a purely formal one. If it is assumed that in about 95% of the eggs there is no crossing over and in the other 5%, for some unspecified reason, there is a great deal of crossing over with the amount of double crossing over that might be expected from it, then if those data are simply added together, the result will be these very high coincidences that I have reported.

I cannot substantiate that hypothesis. There is at least one way in which such an analysis might be approached, namely, to see if there is any clustering of the crossovers. Unfortunately, the output per female is so low that I have not been able to devise any way of attacking the problem from that

point of view. This remains a merely formal sort of suggestion.

In this connection, however, I would like to point out that in studies of interference, the suggested interpretation does represent a type of complication that may arise. If two series of data, in which the coincidences are identical but the frequencies of crossing over in the intervals concerned are different, are added together and the coincidence calculated from the pooled data, the coincidence will always show an increase. This is a simple algebraic result that was demonstrated by Weinstein in 1918. This means that coincidence calculations are very tricky things to work with; that the ordinary calculations, or any other that I have been able to devise, need to be used with caution except in a situation in which there is no variation in crossover frequency. That is a condition very difficult to comply with. It is for that reason, among others, that a good many of us have become suspicious of detailed quantitative analyses of the phenomenon of interference.

In some of the examples which have been described as pseudoalleles in organisms other than *Drosophila*, Dr. Lederberg points out that the interpretation involves the assumption that very short double crossovers have occurred, and that this raises a suspicion that crossovers may not be involved and that mutation will have to be considered as a possible alternative explanation in these cases. I should like to mention that there is on record a model case for the interpretation of some of these situations as mutations—a paper published a good many years ago by Demerec ('26), in which he showed that a particular mutant type known as "reddish" in *Drosophila virilis* did not show any mutation when in the homozygous form but when made heterozygous showed a very considerable mutation frequency. The mutation showed a high correlation with crossing over in the immediate neighborhood, but it was still true that the great majority—about five-sixths of all the mutant chromosomes—had not undergone crossing over in this region. This, I think, is a particularly clear example of the kind of thing

that must be kept in mind — namely, that when an event associated with crossing over is found, it must not be assumed automatically and offhand that it is a consequence of crossing over. It may only, for some reason, be indirectly associated with the possibility of crossing over.

I do not want to take time to present a full opinion of the pseudoalleles in general, but it does seem that they have a bearing on this question of recombination. We are confronted here with a paradox. As you know, the position was as follows a few years ago: Given a mutation,  $a-1$ , and another one independently occurring,  $a-2$ ; suppose these are recessives so that both  $a-1/+$  and  $a-2/+$  are wild type. Now if  $a-1/a-2$  had the  $a$  phenotype, it was concluded that  $a-1$  and  $a-2$  were alleles. This is the classical standard test for allelism. The rule has been that if that test gave the described result, then  $a-1$  and  $a-2$  were at the same locus and crossing over between them was impossible. However, a number of cases have now been noted in which there is a disagreement between these two methods of studying allelism. That is, in a number of cases — an increasing number of them as more work is done — two new types can be recovered from a combination  $a-1/a-2$ . In other words, one can get ordinary crossing over and can show by the use of other marker genes that there is a definite sequence — for example, that  $a-1$  lies to the left of  $a-2$  — in spite of the fact that the phenotype of  $a-1/a-2$  is the recessive one.

There is the further finding that the double heterozygote of the type  $a-1\ a-2/++$  (we must now assume there are two loci here) — i.e., what has been called the *cis* type, is wild type; whereas the *trans* heterozygote ( $a-1+/+a-2$ ) is of the recessive phenotype. This is the phenomenon of pseudoallelism.

The point I should like to insist on here is that, unless both these recombination types ( $a-1$ ,  $a-2$ , and  $++$ ) are recovered, the case for pseudoallelism is incomplete; and in many of the cases that have been reported, only the  $++$  type has been recovered, while the double mutant has not been found. In the absence of that confirmation, I do not think it



is possible to exclude the possibility of mutations of the kind which were described years ago by Demerec.

It would perhaps not be appropriate here to devote more time to this matter of pseudoalleles. The existence of this discrepancy between the two methods of studying allelism has led to a great deal of discussion lately, and to the suggestion that perhaps the classical theory of the gene had better be thrown out completely and a new start made. I do not subscribe to that view to any degree whatever, but I shall not now discuss my reasons.

#### DISCUSSION

PAPAZIAN: I would just like to hear what the reasons were in defense of the discrete gene. Is there anything that you think is good evidence that there are regions of crossing over and regions of genic material that do not cross over?

STURTEVANT: I do not think there is a clear answer on the question of localization of crossing over regions. One of the reasons I do not like the idea that there are no discrete genes is that this means there is a vague indefinite area without any sharp boundaries between one gene and another, and I am not able to visualize that in any physical-chemical terms.

After all, we are dealing with substances of some sort — with material objects. Presumably, they have definite limits. Another point is that in a number of these cases, particularly in the bithorax series studied by Lewis, these separable elements can be shown to have different physiological properties; and one can identify the different loci by looking at the mutant phenotypes associated with them.

WEINSTEIN: I agree with Dr. Sturtevant that we must be careful in interpreting differences in coincidence because they may result from the combination of heterogeneous data. But to prevent possible misunderstanding, it may be well to repeat that the general pattern of variation of coincidence with distance is too marked and too consistent to be accounted for in this way. The rise of inclusive coincidence and of internode frequency is found in several species (including

*Drosophila melanogaster* and *Drosophila virilis*); the subsequent leveling off of inclusive coincidence and fall of internode frequency are found where the distance is sufficiently long (*D. virilis*). In my work with *D. melanogaster* ('18), in which I called attention to the possibility that the combination of heterogeneous data may give rise to spurious differences in coincidence, and in my work with *D. virilis* referred to here, calculations were made not only for the combined data in each cross but for subdivisions of the data — in *D. melanogaster* for each separate culture bottle. The general pattern remained unaffected. This could be further tested by making all the heterozygous parents in each cross genetically identical. Even without such complete homogeneity, however, it is significant that there is agreement between calculations made on total data and on subdivisions. Moreover, it must be borne in mind that the leveling off of inclusive coincidence and the fall of internode frequency for distances long enough to allow crossovers of ranks above 2, are not independent of each other, since the first necessarily implies the second.

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## EVALUATION OF RECOMBINATION THEORY

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As an old-fashioned cytologist I should like to present certain aspects of chromosome structure and behavior which must be considered in any evaluation of the recombination theory. The coiling system in the chromosome cycle is a basic factor in crossing over, and the nature of genic bonds is also significant in chromosome structural changes which occur in nature and can be induced by ionizing radiation.

The mitotic nuclear cycle involves the coiling and uncoiling of the chromosomes. At metaphase the chromosomes are composed of a tightly coiled spiral. The sister chromatids appear to be independently coiled. In *Tradescantia* the number of coils is approximately twenty-five per chromosome. At anaphase the chromosomes may contract further, but as they pass into the resting stage the coiling system is relaxed and the number of coils is reduced. These relic coils are found throughout the resting stage. At early anaphase the new coiling system begins and, eventually, the relic coils are replaced by new minor coils. During the transition there is some coiling of sister chromatids about each other in the form of relational coiling, but by metaphase this relational coiling has usually been eliminated and the sister chromatids are separate and parallel. At no time in the mitotic cycle are the chromosomes free from coiling.

In meiosis, on the other hand, the coiling system differs from that in mitosis in several essential respects. The pre-meiotic nuclei increase in size until at meiotic prophase they are considerably larger than those in mitosis. The relic coils largely disappear and the chromosomes are free to pair. Pair-

ing is facilitated by the polarity of chromosomes, with the centromeres oriented toward one side of the nucleus. The relative freedom from relic coiling permits an intimate and very precise pairing of homologous chromosomes. This pairing must be even more precise than the relatively gross cytological observations would indicate, since crossing over must occur at the molecular level.

The molecular coiling system of deoxyribonucleic acid (DNA) described by Watson may be the basis for the cytological coiling system. Even the microscope observations indicate coils within coils at meiosis and it is not unreasonable to envisage a microscopic coiling system as initiated by a molecular coiling system. However the direction of coiling in both mitotic and meiotic chromosomes is variable in direction of coiling.

The intimate association of homologous chromosomes is limited to meiotic prophase owing to the precocity of nuclear development in relation to chromosome development. The actual crossing over between chromatids may also be related to the coiling system. According to Darlington, it is the relational coiling of homologous chromosomes and sister chromatids which imposes the stress needed to break the chromatids and permit a new association of chromatids. This assumption is in accord with the cytological and genetic observations of interference. Any theory of crossing over must conform to the facts of interference. Such a gross mechanism of breakage and reunion is, however, difficult to reconcile with the precision of genetic crossing over.

Unlike the chromosome recombinations induced by radiation and certain chemical agents, the crossovers are always polarized and progressive from the proximal to the distal ends of the chromosome arms. Lateral fusions which give rise to dicentric bridges seldom if ever occur in normal meiosis. Yet the breakage and reunion of chromosomes induced by ionizing radiation show no evidence of polarity of the bonds between genic elements. An induced inversion is usually viable and functions as well as the original chromosome. These



induced breaks, whether in sister chromatids or in different chromosomes, may also reunite laterally to give rise to dicentric, or progressively to give rise to exchanges and translocations.

In regard to stress factors involved in chromosome breakage and recombination there does seem to be some similarity in crossing over and artificial induction of chromosome recombination. Some stress factor appears to be involved in meiosis to account for interference. In recombinations induced by ionizing radiation there is considerable evidence which indicates that chromosome stress and movement are important factors in the production of chromosome aberrations. In the first place, the chromosomes of a given species become more sensitive to induced aberrations as they progress from the relatively inactive state in the resting cell to the more active prophase with maximum sensitivity at metaphase or early anaphase. It is also possible to augment the frequency of induced aberrations by centrifuging the cells while under irradiation; or as Conger has done, by subjecting them to ultrasonic radiation during exposure to X rays. Presumably these stress factors, both under normal conditions and under experimental control, shift the broken ends of the chromosomes out of alignment, thus preventing restitution and favoring illegitimate unions of broken ends which result in visible chromosome aberrations.

Various attempts have been made to account for random crossing over between the chromatids of homologous chromosomes at meiosis. The suggestion by Schwartz invokes sister-chromatid crossovers to account for the random crossing over. It is assumed that crossing over between homologous chromosomes is always limited to the newly formed chromatids in each of the homologs, so that sister-strand crossing over would be necessary to produce random recombination. If sister-strand crossovers, involving the old and new chromatids, are possible it would be reasonable to suppose that crossing over between the chromatids of the two homologous chromosomes need not be limited to the newly formed chromatids.



Nor does this modified Belling's theory provide any adequate basis for genetic and cytological interference.

The nature of the broken ends of chromosomes may be of considerable significance in several respects. In the first place there must be bonds which can be broken and joined with no effect on the adjacent genic centers. Neither crossing over nor induced chromosome breakage normally results in genic changes. Watson and others have suggested that the actual breaks do not occur in the DNA molecules, but in the linkage between them.

Once a chromosome bond is broken it remains in an unstable condition and unites readily with other broken ends. It cannot normally constitute a normal end of a chromosome and behave as a telomere. The unstable condition of these broken ends may be of some significance in transduction. An interstitial chromosome deletion usually, if not always, forms a ring chromosome fragment by the union of the two broken ends. If a transduction involves anything comparable to the insertion of a chromosome segment, two questions arise. How are the ends of the fragment prevented from uniting to form a ring, and how is the break induced in the recipient chromosome?

If, on the other hand, the transduction is effected by a lateral association of the contributed DNA with the recipient chromosome and alters the normal gene segment, it is difficult to understand gene stability in hybrids where unlike genes are in intimate association at meiosis. Perhaps transduction involves a mechanism such as the activators found in *Zea* by McClintock, the nature of which is not yet known.

I hesitate to discuss some of the agents which modify X-ray-induced mutations, and particularly the oxygen effect, since this controversy involves several of my former students. Other modifying agents indicate that the modifiers may act quite indirectly. With all of the various modifying agents available, both physical and chemical, we should be able to arrive at a more precise understanding of the nature of chromosome breaks and reunions.

The induced chromosome aberrations should also lead to a better understanding of chromosome recombination in nature. Carson has pointed out the significance of structural chromosome alterations in speciation and evolution. These changes may be even more significant than we realize if introgressive hybridization is as prevalent as Edgar Anderson believes it is. A few segments of chromosome material introduced from one species to another not only results in variation directly, but could be followed by further variation resulting from occasional crossing over in these small segments in heterozygous combinations. The role of chromosome recombination is an important factor in speciation and evolution, and the radiation work has led to a better understanding of the mechanisms leading to inversions, translocations, and deletions.

The problems raised at this symposium can be solved to some extent by studies of gross chromosome structure and behavior, but the ultimate solution must rest on analyses at molecular levels. In some respects these levels may underlie the microscopic observations and, particularly in the coiling cycle, may be similar. Since in the higher plants there are differences in chromosome size of the order of a hundredfold, perhaps the chromosomes which we are unable to detect in certain microorganisms are structurally similar to the larger chromosomes, and, in turn, the molecular structures may provide the pattern for coiling cycle and behavior of the visible chromosome.

I do not know why the cytogeneticists of the Paleozoic Age of modern biology should be asked to summarize this ultra-modern symposium. We hope that the new results can be reconciled with the long-established facts of genetics and cytology. It is encouraging to see that new tools for investigation have been developed which should permit a more critical analysis of the various problems of cytology, genetics, and evolution.



# UNRAVELING THE CHROMOSOMES

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EIGHT FIGURES

## THE WIDENING SCOPE OF GENETICS

The way of the summarizer is hard. Speakers in this symposium have told us many things, and told them explicitly; and it would be impossible, in the space allotted, to summarize all we have heard and to explore all its connections with biological problems and theories.

One of the noteworthy things about this conference is that it has included researches not only on the classical material of genetics but on microorganisms as well. A short time ago this would have been impossible, partly because the genetic mechanisms of microorganisms were unknown, and partly because many scientists thought that such mechanisms did not exist. It is well to recall, however, that this confusion of thought did not characterize the great biologists of the nineteenth century. Darwin (1875) said that hereditary units "can hardly differ much in nature from the lowest and simplest organisms," under which he included viruses; and Weismann ('02, '04) expressed a similar opinion.

Genetics has expanded not only to include more kinds of organisms; but also, in another direction, to include, or at any rate to connect with, physiological and embryological processes. Geneticists used to be accused of regarding the gene as a kind of billiard ball, with no structure that would allow for chemical activity. It is true that some biologists drew a distinction between genetic and physiological traits,

<sup>1</sup> Working on grant from the Commonwealth Fund.

but the leaders of genetic thought were not among them. Bateson, both before and after he introduced the term "genetics," referred to the science as the physiology of heredity and variation; and he spoke of it as "standing next beside, and looking constantly for support to, physiological chemistry" ('07). He suggested that "the operations of some [hereditary] units are in an essential way carried out by the formation of definite substances acting as ferments" ('09). And Morgan, Sturtevant, Muller, and Bridges ('15) wrote: "On the factorial hypothesis the factors are conceived as chemical materials in the egg, which, like all chemical bodies, have definite chemical composition."

Nevertheless, genetics seemed so isolated that it was not treated in D'Arcy W. Thompson's book "On Growth and Form" ('17, '42), which undertook a synthesis of embryology, morphology, and physiology from the standpoint of mathematics and physics. The omission is all the more significant because Thompson was interested in problems that are mathematically related to the coiling and unraveling of the chromosomes. He discussed the geometry of spirals and helices in shells and plants; and in another work ('40) he wrote about a game called "trick-in-the-loop" in which a strap is coiled in such a way that a wooden pin inserted into it is not held in any coil when the strap is loosened out.

#### COILING AND REPLICATION

Mitosis and meiosis may be regarded as varieties of trick-in-the-loop in which there is no pin, but in which two or four strands are unraveled from each other. A somewhat similar situation occurs in some other cell organs, the flagellar bands, which may number from two to sixty, in certain protozoa, and which are transmitted to daughter cells in cell division (Cleveland, '53). It has now appeared that a variety of trick-in-the-loop must be involved in the replication of molecules of nucleic acids and proteins.

In all these cases the geometric problem is the same — to separate the strands. But the way in which the separation is



to be achieved must differ according to conditions — rigidity, friction, the number of points at which the separating forces are applied, and whether or not each strand remains intact.

The separation is easiest when rigidity and friction are small, and each strand is pulled at only one point. For then the convolutions can unwind and slide over each other. Unwinding occurs in the flagellar bands mentioned. In chromosomes, there have been differences of opinion as to whether separation is accomplished only by uncoiling; a summary and a bibliography are given in the review of Manton ('50). In deoxyribonucleic acid (DNA), it is not known how the strands separate.

If the separating forces are applied at more than one point in each strand, for example at both ends, there may be interlocking even in the absence of rigidity and friction. If equal parallel forces are applied at all points of each strand, the situation is the same as if the strands were rigid.

The geometric problem of unraveling rigid strands is more easily understood, I think, from models, which can be felt as well as seen, than from pictures, which appeal only to the sight, or from descriptions, which appeal only to the imagination. Models can be made by winding two strips or ribbons of different colors around a cylinder. It helps if the cylinder is transparent; a tube of glass or lucite can be used. And instead of two separate strips, we can use one strip of rubber colored differently on the two sides.

If the coiling is all in the same direction, as in figure 1 A, then if we view the model at right angles to the long axis, we can see both colors, no matter which side we look from. If we should try to pull either color toward ourselves, it would interlock with the other. This means that if a chromatid or a molecule replicates on one surface — the same surface throughout its entire length — and if it coils in this way, the two resulting chromatids or molecules would interlock if they began to move away from each other laterally. The same would be true if replication were along an edge, provided that it is the same edge throughout the entire length.

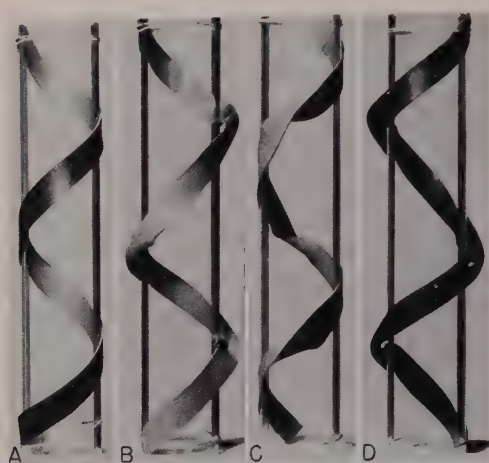


FIG. 1

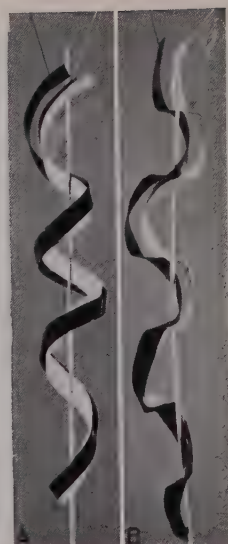


FIG. 2

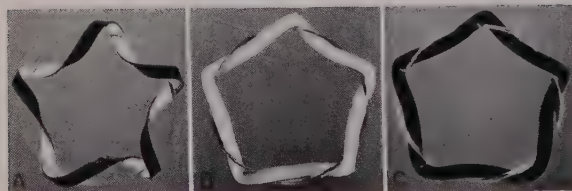


FIG. 3



FIG. 4

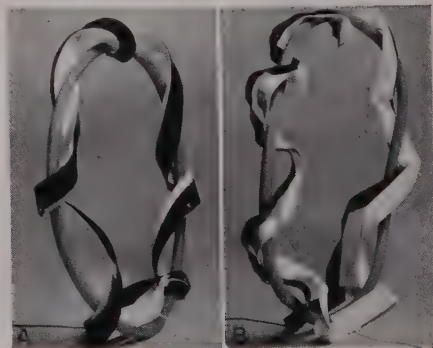


FIG. 5

Fig. 1 Rubber strip, dark on one side, light on the other, coiled around a lucite tube. In A, the coiling is constant in direction. In B, C, D, which are viewed at intervals of  $90^\circ$ , the coiling is reversed at each half-twist.

Fig. 2 Aluminum strips coiled around a rod. In A, the coiling is constant in direction; in B, it is reversed at each half-twist.

Fig. 3 Rubber strip, dark on one side and light on the other, coiled around a lucite ring. In A, the coiling is constant in direction. In B and C, which are viewed from opposite sides, it is reversed at each half-twist.

Fig. 4 Same models as in figure 3 viewed at an angle. In A, the coiling is constant in direction; in B, it is reversed at each half-twist.

Fig. 5 Aluminum strips coiled around a circular rubber tube. In A, the coiling is constant in direction. In B, it is reversed at each half-twist.

The separation can be achieved, as is well known, if the coiling is not all in one direction, but if each clockwise half-turn is followed by a half-turn that is counterclockwise (fig. 1 B, C, D). In this case it is possible to get a view that shows only one color and another view that shows only the other color. Therefore these two colors can be pulled apart without interlocking.

These two types of coiling can also be illustrated by models made of aluminum strips (fig. 2). An advantage of these is that they retain their shape while being handled.

These relations still hold if the strips are bent in a circle, as in ring chromosomes. The rubber strips can be wound around a lucite ring (figs. 3, 4). The coiled aluminum strips can be bent till the ends touch, and a wire or a piece of thin rubber tubing can be inserted to mark the axis around which they coil (fig. 5). Where the coiling is all in one direction, no separation is possible; in fact, now there is interlocking even in the absence of rigidity and friction. Where the coiling is reversed at each half-twist, the strips can be separated; and if there is no rigidity or friction, the coils need not alternate, provided that the total number of coils in one direction is equal to the total number in the other direction.

What has been said would apply to the relatively simple case of mitosis, where there are two chromatids that do not cross over, or to the replication of a chain molecule. In meiosis the situation is more complicated because there are four strands. In addition, there may be crossing over, which may make separation easier by preventing interlocking or may introduce interlocking where it would not otherwise exist. Crossing over adds another complication: either strand of one chromosome must be able to touch either strand of the other at any level at which exchange takes place.

The general result of these complications is that, for many cases at least, unraveling requires nonrigidity, or sister-strand exchanges, or both.

A set of rules for the genetic game of trick-in-the-loop would be useful, and would answer the question asked by Dr. Crick (this conference): What are the phenomena in crossing over to be explained? The phenomena to be explained, or the rules to be obeyed, are essentially, I think, as follows, though additions may be made necessary by further research.

1. In each chromosome the genes are arranged in a linear series.

2. Normally, corresponding genes occupy corresponding positions, and corresponding groups of genes are exchanged in crossing over.

3. Exchanges may occur at more than one level.

4. Exchanges occur when each chromosome consists of two chromatids.

5. At any level, crossing over is limited to two chromatids, one from each chromosome.

6. At any level, either chromatid of one chromosome is equally likely to cross over with either chromatid of the other chromosome.

7. The two chromatids that cross over at one level do not decide which two cross over at other levels; that is, there is random recurrence of crossing over (no chromatid interference).

8. There is regional interference. An exchange interferes with exchanges at neighboring levels, and this interference decreases with distance and finally vanishes. This means that inclusive coincidence increases from 0 to 1 and remains at the latter figure; and that internode frequency rises from 0 to 1 or less, and then, if there are exchanges at more than two levels, decreases again.

9. Either exchanges do not occur between sister chromatids; or if they do, they do not interfere with exchanges between homologous chromatids at other levels.

10. In the exceptional cases where crossing over is unequal, exchanges between sister chromatids occur rarely if at all.



## CHROMATID INTERFERENCE

It has been inferred from both cytological and genetic evidence that the two chromatids which cross over at one level determine to some extent which two cross over at other levels.

The cytological evidence is faced with two difficulties. In the first place, in tetrads having more than one chiasma, it is not always possible to tell whether two, three, or four chromatids are involved; though some workers have thought that the uncertain cases are not sufficiently numerous to prevent significant conclusions. And in the second place, it has been shown that a chiasma does not necessarily represent a genetic exchange (Cooper, '49).

The genetic evidence that has been relied on most is that from *Neurospora*, where all four chromatids are recovered and their relative positions during meiosis are known. This evidence, however, has left some investigators unconvinced because of possible uncertainties in the classification of characters and the interpretation of results. Some of the difficulties were mentioned by Dr. Perkins in his talk at this conference.

In *Drosophila* the evidence from ordinary diploids, from attached X's, and from ring chromosomes is consistent with the absence of chromatid interference (Weinstein, '28, '32a, '36; Emerson and Beadle, '33; Beadle and Emerson, '35; Emerson and Rhoades, '33; L. V. Morgan, '33). The deviations are for the most part explicable as arising from differential viability or errors of sampling; though Bonnier and Nordenskiöld ('37) have interpreted their results as indicating chromatid interference.

The problem can be dealt with by means of a set of formulas (Weinstein, '38a, b, '48) that express the relations between tetrad frequencies and (1) recombination values, (2) chromatid frequencies, and (3) homozygosis in attached X's, in terms of the frequencies of regressive (2-strand), progressive (3-strand), and digressive (4-strand) exchanges. If the relative frequencies of regressive, progressive, and digressive exchanges are represented by F, G, and H respectively, the



recombination value depends on the magnitudes of F and H, whereas homozygosis in attached X's depends on G.

1. The recombination value falls short of 50% if F exceeds H, and may rise above 50% if F is less than H. If F is equal to H, the recombination value rises toward and may reach 50%. Since in all organisms that have been studied, including all species of *Drosophila*, the recombination value approaches but never exceeds 50% in any undoubted case, the evidence points to an equality of F and H (see also Carter and Robertson, '52).

2. When tetrad frequencies are calculated from observed chromatid frequencies, negative numbers result if there is an excess of F over H, and also if there is any but a slight excess of H over F. My calculations have been made on extensive data in both *Drosophila melanogaster* and *Drosophila virilis*. Since negative frequencies are impossible, this evidence also points to an equality of regressives and digressives, or at most to a slight excess of digressives.

3. If then we tentatively accept the tetrad frequencies calculated on the assumption that F is equal to H, and use them to calculate homozygosis in attached X's for various values of G, we can discover what values of G lead to results in best agreement with experiment. Thus F, G, and H will all be evaluated.

Homozygosis curves in *D. melanogaster* for various values of G are shown in figure 6. The curves almost coincide in the proximal part of the chromosome, from *bobbed* to *miniature*; because for a region as short as this, crossovers are practically all singles, and chromatid interference, if there is any, scarcely comes into play. As the distance becomes longer, the number of multiple exchanges increases, and the curves diverge.

The observed values of homozygosis from experiments of various workers are indicated by the points on the figure (fig. 6). In the region from *bobbed* to *miniature* the observed frequencies for mutant genes tend to lie below the curve, whereas those for wild-type alleles tend to lie on or above

the curve. This suggests that deviations from the curve are due to differential viability. In any event, in this part of the graph, as has been explained, almost all the exchanges are singles, so that chromatid interference is scarcely involved.

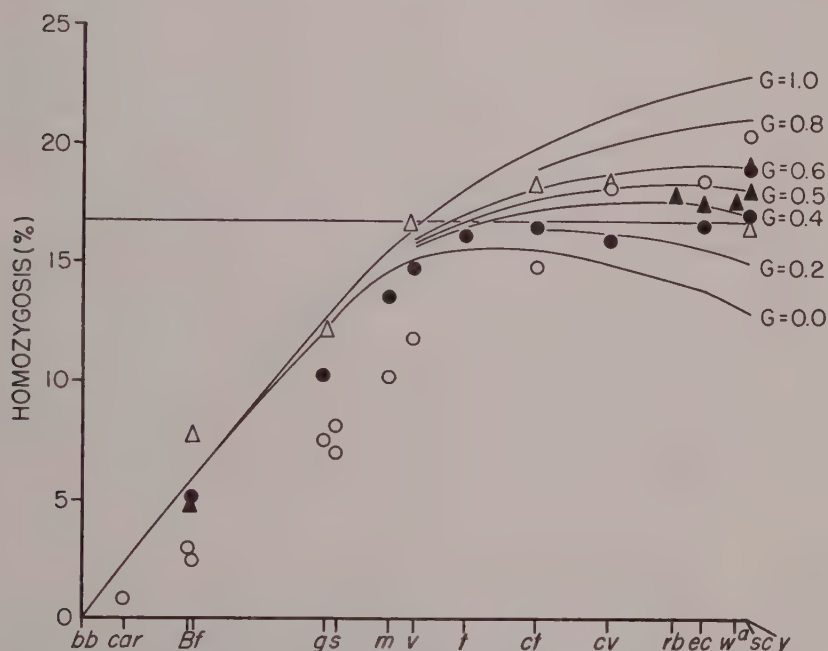


Fig. 6 Proportions of homozygosis for one gene of a pair in offspring of heterozygous attached-X females. The abscissa indicates distance from the spindle fibre. The curves are drawn for different values of  $G$ , the proportion of progressive (3-strand) exchanges, on the basis of tetrad frequencies calculated by Weinstein ('36, table 8, column C) from data of Bridges (Morgan, Bridges, and Schultz, '35). The observed homozygosis values are from Anderson ('25), L. V. Morgan ('25), and Sturtevant ('31), summarized by Sturtevant (solid circles); Emerson and Beadle ('33) and Beadle and Emerson ('35) (open circles); Rhoades ('31) (solid triangles); Bonnier and Nordenskiöld ('37) (open triangles).

In the region where the theoretical curves do not coincide, from *vermillion* to the distal end, the observed values are about equally numerous above and below the curve for  $G = 0.5$ , and tend to lie between the curves for  $G = 0.4$  and  $G = 0.6$ . In this region (as throughout the chromosome) the observed values must differ from each other and from the calculated

values not only on account of differential viability but also because of differences in crossing over in the different experiments.

When all these facts are taken into account, the observed homozygosis agrees with the calculated value for  $G = 0.5$ . Since  $F + G + H = 1$  by definition, and since  $F = H$  on the basis of recombination and chromatid frequencies, it follows that  $F$  and  $H$  are each equal to 0.25. This ratio,  $\frac{1}{4}$  regressives:  $\frac{1}{2}$  progressives:  $\frac{1}{4}$  digressives, is what is meant by random recurrence of crossing over, or the absence of chromatid interference.

In these calculations, and in others which yield similar results, it has been assumed that  $F$ ,  $G$ , and  $H$  are constant throughout the chromosome; that is, that they do not vary with region or distance. Calculations now being made, but not yet complete, in which  $F$ ,  $G$ , and  $H$  are allowed to vary with distance, also rule out chromatid interference, at least for the great majority of cases, because it would lead to impossible results.<sup>2</sup>

#### REGIONAL INTERFERENCE, COINCIDENCE, AND INTERNODE FREQUENCY

Since there has been misunderstanding about different types of coincidence and their meaning, a few necessary distinctions will be mentioned briefly. I shall refer to the regions whose coincidence is being measured as nodal regions, and to a crossover in two nodal regions as a binodal.

If  $N$ ,  $A$ ,  $B$ , and  $M$  are respectively the numbers of all individuals, of crossovers in the first nodal region, of crossovers in the second nodal region, and of binodals, the coincidence is the ratio of the actual proportion of binodals to the proportion expected on chance,

$$\frac{M/N}{(A/N)(B/N)} = \frac{MN}{AB}.$$

<sup>2</sup> These calculations are being performed on a UNIVAC electronic computer. I wish to express my thanks to Remington Rand, Inc., Philadelphia, and to Dr. John W. Mauchly, Mr. William Turanski, and Mr. Bevier Hasbrouck of the staff of the Eckert-Mauchly Division of Remington Rand, Inc.

As the nodal regions become more widely separated, coincidence increases from 0 to 1; that is, an exchange interferes with neighboring exchanges, and the interference decreases and finally vanishes as the intermediate distance lengthens (Sturtevant, '13, '15; Bridges, '15; Muller, '16). The coincidence is a measure of interference, or rather of the lack of it. In these early experiments there were no exchanges of ranks above 2, hence no binodal was a crossover in the intermediate region, and the coincidence showed that long internodes are more frequent than short ones.

That the coincidence was a measure of internode frequency can be shown as follows (see fig. 7). Suppose that one nodal region consists of two units,  $a_1$  and  $a_2$ , and the other of three units,  $b_1$ ,  $b_2$ , and  $b_3$ . Then six classes of internodes are possible according to whether the exchanges involve regions  $a_1b_1$ ,  $a_1b_2$ ,  $a_1b_3$ ,  $a_2b_1$ ,  $a_2b_2$ , or  $a_2b_3$ . These internodes will differ little in

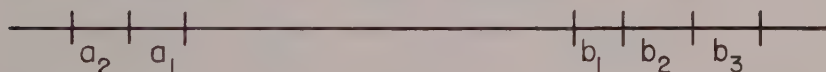


Fig. 7

length if the nodal regions are short in comparison to the intermediate region, and they will occur with approximately equal frequencies if the variation of coincidence with distance is gradual. If in one experiment the nodal regions are  $a_1$  and  $b_1$ , in another  $a_1$  and  $b_1 + b_2$ , and in a third  $a_1 + a_2$  and  $b_1 + b_2 + b_3$ , the respective frequencies of internodes will be in the ratio 1:2:6. To eliminate the influence of the lengths of the nodal regions, the second frequency must be divided by 2, and the third by 6. In general, the frequency of internodes is proportional to the product of the genetic lengths of the nodal regions. If it is divided by the product, the corrected frequency is

$$\frac{M/N}{(A/N)(B/N)} = \frac{MN}{AB},$$

which is the coincidence.

In my experiments with *D. melanogaster* ('18) the nodal regions were so widely separated that some binodals were

also crossovers in the intermediate region. Hence it was necessary to distinguish between two types of coincidence. When interference is measured, the term  $M$  must include all binodals; when internodes are counted, the term  $M$  must include only those binodals that are not crossovers in the intermediate region. In the latter case the term may be written  $M_0$ . Thus the coincidence fraction assumes two different forms. Inclusive coincidence,

$$\frac{M/N}{(A/N)(B/N)} = \frac{MN}{AB},$$

is a measure of interference. Select coincidence,

$$\frac{M_0/N}{(A/N)(B/N)} = \frac{M_0N}{AB},$$

is a measure of internode frequency, and I shall refer to it as internode frequency to avoid misunderstanding.

In my 1918 experiments both types of coincidence rose to about 1 and then decreased. I suggested however (pp. 150–151) that further experiments might show that inclusive coincidence remains at 1, but that even in this case the internode frequency must decline if some binodals are also crossovers in the intermediate region.

This turned out to be the situation in the X chromosome of *D. virilis*, which was used in my subsequent experiments ('32b, '35) because it is genetically longer than the *D. melanogaster* X. Figure 8 shows the results in a six-point cross covering 100 units in *D. virilis*. The inclusive coincidence rises to 1 and remains at that level; the internode frequency rises, though not so high, and then declines to 0.55 for chromatids and to 0.2 for tetrads. The absence of interference beyond about 40 units explains why internodes tend to be of that length; for an internode longer than 40 units tends to break into shorter ones because exchanges within it are interfered with to a lesser extent, or not at all, by the terminal exchanges.

These results in *D. virilis*, based on 33,048 individuals, are statistically significant and are not caused by differential viability. They are supported by a five-point experiment



covering the same distance and involving almost as many flies, and by numerous other data.

In *D. melanogaster* I have made calculations on the published data of various workers and on extensive unpublished data of Dr. C. B. Bridges, which he had kindly placed at my disposal. The results in this species are not always consistent. Sometimes, as in *D. virilis*, inclusive coincidence levels off at about 1, whereas internode frequency declines; sometimes

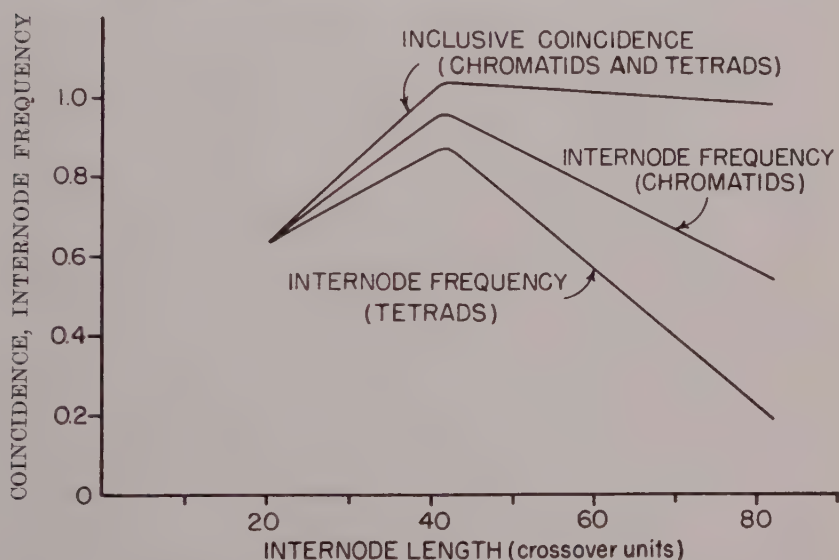


Fig. 8 Coincidence and internode frequency in six-point cross (*sepia cross-veinless singed diminutive triangle rugose*) in *Drosophila virilis*.

both decline. Such irregularities, if not due to differential viability or sampling deviation, may be connected with the fact that some of the regions studied are close to the spindle fibre, and that in the autosomes the spindle fibre attachment is median.

The variation of the results in *D. melanogaster*, and other causes as well, have given rise to misunderstanding. The term "interference," or "index of interference," had been used for the reciprocal of the coincidence fraction before it had become necessary to distinguish different types of coincidence;

and for a time it continued to be used as the reciprocal sometimes of one type, sometimes of the other. No harm would have resulted if the sense in which the term was used had always been stated. Again, the concept of partial coincidence, which was proposed by Muller ('25) and which may be expressed as

$$\frac{M_o/N_o}{(A_o/N_o)(B_o/N_o)} = \frac{M_o N_o}{A_o B_o}$$

to indicate that crossovers in the intermediate region are excluded from every term, was by some writers confused with internode frequency. Furthermore, it was sometimes assumed that only one type of coincidence can be correct, and that it must apply to all cases.

As a result, some invalid criticisms were put forward. The conclusion that internode frequency declines for widely separated regions was objected to on three grounds: (1) that the decline is due to the exclusion of crossovers in the intermediate region from the binodal class, (2) that the decline is due to the nonexclusion of crossovers in the intermediate region from the binodal class; and (3) that there is no decline. Schweitzer, who in 1934 objected (on all three grounds) to the formula for select coincidence or internode frequency, in 1935 proposed a formula (called by him "corrected doubles") which for any cross is merely internode frequency multiplied by a constant, so that the relative values remain unchanged.<sup>3</sup> Stevens ('36) put forward a type of coincidence which he apparently thought to be new, and he criticized previous workers for not having used it; yet his formula is identical with inclusive coincidence, which had been used by at least three previous workers (Bridges, '15; Muller, '16; Weinstein, '18) and its reciprocal (index of interference) had been used by Sturtevant ('15).

Much of the misunderstanding could have been avoided by an acquaintance with the earlier investigations. The papers

<sup>3</sup> Stevens' statement ('36) that the formula for "corrected doubles" is inclusive coincidence multiplied by a constant arises from a misunderstanding of Schweitzer's explanation.

of Sturtevant, Bridges, and Muller are classics in this field; and a geneticist has done me the honor of applying this term to my paper. He did not define the term, but there is a definition which, I think, fits the case: a classic, as we have all heard, is a work that is often referred to and never read.

#### THE QUESTION OF SISTER-STRAND CROSSING OVER

One way in which coincidence enters into the problem of the mechanism of crossing over is in connection with the question whether exchanges occur between sister strands. If, at the time of crossing over, sister chromatids have become completely independent of each other and if their relation to each other is the same as to other chromatids, we might expect sister-strand exchanges to interfere with homologous-strand exchanges at other levels to the same extent that the latter interfere with one another. But experiments on crossing over show that sister-strand exchanges cannot occur if they interfere with homologous-strand exchanges to this extent (Weinstein, '28, '32a, c, '36, '38a; L. V. Morgan, '33; Emerson and Beadle, '33; Beadle and Emerson, '35). And this is in harmony with the fact that no sister-strand exchanges have been found in unequal crossing over, where they can be observed directly (Sturtevant, '25, '28; Muller and Weinstein, '32; Sturtevant and Beadle, '36).

On the other hand, there is evidence (Weinstein, '32a, c, '36) that (1) sister chromatids are not independent of each other when crossing over takes place, (2) their relation to each other is different from their relation to the other chromatids, and (3) sister-strand exchanges therefore need not interfere with exchanges between homologous strands. The occurrence of sister-strand crossing over under these conditions is consistent with experiments on normal crossing over, and is not necessarily contradicted by the absence or rarity of sister-strand exchanges when crossing over is unequal. For sister genes, whether they arise by division or by the synthesis of one under the influence of the other, must at first lie close together; and hence it may be impossible or difficult for one

to shift its position so as to lie alongside the gene at the next locus, even if that gene is a duplicate. Thus sister chromatids may be unable to cross over unequally. But homologous chromatids are not so close together to start with, and a gene in one can be brought alongside either of two duplicate genes in the other, so that unequal exchanges can occur.

The problem of sister-strand crossing over is also connected with that of chromatid interference. Two questions are involved, as Dr. Perkins said (this symposium): (1) Can sister-strand exchanges produce the random ratio of 1 regressive: 2 progressives: 1 digressive from a nonrandom ratio?

TABLE 1

*How the relation of homologous-strand exchanges to one another is altered by sister-strand exchanges in the intermediate region*

NO. OF INTERMEDIATE SISTER-STRAND EXCHANGES IN THE TWO CHROMOSOMES OF THE TETRAD	TYPE OF HOMOLOGOUS-STRAND EXCHANGE IN THE ABSENCE OF SISTER-STRAND EXCHANGES		
	A	B	C
	REGRESSIVE	PROGRESSIVE	DIGRESSIVE
Even (or 0) and even (or 0)	Regressive	Progressive	Digressive
Even (or 0) and odd	Progressive	{ Regressive Digressive	Progressive
Odd and odd	Digressive	Progressive	Regressive

(2) Can they produce a nonrandom from a random ratio? Table 1 can help in answering these questions.

Column A shows, as has been pointed out by several workers (Sax, '32; Weinstein, '32a, c, '36; Lindegren and Lindegren, '37; Schwartz, '53) that a theory such as Belling's ('31), which allows only regressive exchanges, can be made to yield other types by means of sister-strand crossing over. Columns B and C show that progressives and digressives can also be changed in this way. In each column the result depends on whether the number of sister-strand exchanges in the intermediate region is even in both chromosomes, or even in one and odd in the other, or odd in both.

If the number of sister-strand exchanges is equally likely to be even and odd in any tetrad and in either chromosome of



a tetrad (as may be expected if the exchanges are numerous, though not necessarily only on this condition), the ratio of combinations will be 1 even-even: 2 even-odd: 1 odd-odd; and this will also be the ratio of regressives, progressives, and digressives in each column. Hence it will be the ratio in all the columns taken together, regardless of the ratio of the three columns to one another.

The reverse effect can also be produced. If columns A, B, and C are in the ratio 1: 2: 1, a different ratio can be brought about among the resulting types by sister-strand exchanges, provided that the sister-strand exchanges are not distributed at random with respect to homologous-strand exchanges. This can come about in two ways.

1. The ratio of even-even, even-odd, and odd-odd combinations may not be the same in all three columns. For example, if sister-strand exchanges occur only in column C, then some digressives will be changed into other types, and the resultant deficiency of digressives will not be compensated for in other columns. It should be noted that it is neither necessary nor sufficient that the ratio of combinations deviate from 1: 2: 1 in all columns; but it must not deviate in the same way in all columns.

2. Even and odd numbers of sister-strand exchanges may not be equally likely in the two chromosomes of a tetrad. For example, let us suppose that in every tetrad one sister-strand exchange occurs in the intermediate region in one chromosome and none in the other. In a progressive tetrad there might be a tendency for the sister-strand exchange to occur in one chromosome rather than in the other. This would produce an excess of regressives or of digressives, which would not be compensated for elsewhere. For in column A there is no reason why the sister-strand exchange should be in one chromosome rather than in the other; and even if such a tendency did exist, the result would be a progressive in either case. The same is true of column C. In this example, any odd number may be substituted for 1 and any even number for 0.



These examples of how sister-strand exchanges can cause a deviation from a 1:2:1 ratio of regressives, progressives, and digressives have been given not as probable cases but as illustrations of the kind of situation that would be necessary if sister-strand exchanges are to change the random 1:2:1 ratio into a nonrandom ratio.

If there is crossing over between sister chromatids, some indication of it might be expected in the chemical structure of the genetic material. In Watson and Crick's ('53) model of the DNA molecule, the two helices suggest sister strands. But there is the complication that the two helices are not identical, since they run in opposite directions. Watson and Crick have pointed out the need for more knowledge about such matters as whether the DNA helices extend continuously through the entire length of the chromosome. And information is also needed about what parts may be played by other constituents.

That the study of crossing over should lead to specific questions which are chemical and which are closely related to the problem of how the genes produce the characters and how they mutate, shows how far the subject has progressed. In the earliest days it was arithmetical, dealing with numerical ratios. It became geometric with the location of the genes in the chromosomes and the construction of chromosome maps. Questions of the forces involved in crossing over and interference connected the subject with mechanics; the effects of heat and radiation connected it with other branches of physics. And now that investigations of the chemical constitution of the chromosomes have begun to tie up with crossing over, we are in the field of physical chemistry.

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# THE OXYGEN EFFECT ON RADIATION-INDUCED CHROMOSOME ABERRATIONS: BREAKAGE- VERSUS-RECOMBINATION HYPOTHESES <sup>1</sup>

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Before consideration of the particular problem under discussion, it seems desirable to review briefly the nature of the evidence indicating an effect of oxygen on the production of chromosome aberrations by ionizing radiations. In doing this, an attempt will be made to establish areas in which agreement exists before discussing points where there are differences of interpretation.

An effect of oxygen removal on radiosensitivity as measured by chromosome aberration production with X rays was demonstrated in *Vicia faba* root tips by Thoday and Read ('47). Their observations were soon confirmed and extended by other investigators. An effect of oxygen on the radiosensitivity of chromosomes to X rays is now thought to be a universal phenomenon. Indeed, it appears that damage by X rays to biological systems generally is influenced by oxygen tension. Thoday and Read ('49) also showed that there is little or no oxygen effect when  $\alpha$  radiation is used, and these observations have also been confirmed and extended by others. The ensuing discussion will deal principally with results obtained from the irradiation of *Tradescantia* chromosomes, but certain references will also be made to observations on other materials.

<sup>1</sup>The experimental work reported here has been supported in part by a research contract with the Atomic Energy Commission at Yale University.

In considering the evidence for and interpretations of the oxygen effect, two questions may be immediately posed. (1) Is the removal of oxygen, rather than some other accompanying condition, actually responsible for the observed decrease in radiosensitivity? In answer to this question there appears to be general agreement that oxygen is the effective agent. Irradiation in a vacuum, as well as the substitution of several different gases for oxygen, give substantially the same result (Thoday and Read, '47; Giles and Riley, '49). A regular relation between increasing oxygen tension and aberration frequencies has also been demonstrated (Giles and Beatty, '50). Furthermore, certain instances of chemical protection against radiation damage in the killing of bacteria have been demonstrated to result from the removal of oxygen from the cellular environment (Hollaender and Stapleton, '53). The second question is: (2) When does the oxygen effect occur relative to the time of X-ray exposure? This question is particularly pertinent to our major problem, since in *Tradescantia* as well as in most other organisms, it is evident that the breakage of chromosomes by radiation and the recombination of the resulting broken ends are clearly separable processes, even though recombination may follow breakage relatively rapidly, as the evidence indicates for *Tradescantia*. It should be noted here that, although the occurrence of relatively rapid recombination (restitution and reunion) in *Tradescantia* has been recently questioned (Lane, '51), this report has not been supported by several subsequent independent studies (Sax and Luippold, '52; de Serres and Giles, '53; Giles, de Serres, and Beatty, '53; Steffensen and Arnason, '54). The experimental data of Sax and others, as analyzed by Lea ('46), indicate that the average time during which the majority of breaks remain open and capable of reunion is of the order of 4 minutes, although some breaks may remain open for much longer times. Hence, in *Tradescantia* it is possible to achieve a reasonably effective temporal separation of the breakage and recombination processes and thus to test for an effect of oxygen as such on the two separate

reactions. The data resulting from experiments designed to obtain this type of information are shown in table 1. It is clear from these results that oxygen has an effect on aberration yield *only* when it is present during irradiation. Furthermore, there is little if any lag between the introduction of oxygen and its effect. These and other experiments in

TABLE 1

*Experiments concerning the effect of the presence or absence of oxygen during and after X irradiation on the frequency of chromosome aberrations induced in Tradescantia microspores*

All exposures 300 r at 300 r/minute  
(Data from Giles and Riley, '50; Giles, '52a)

SERIES NUMBER	EXPOSURE CONDITIONS	POSTTREATMENT CONDITIONS	NO. OF INTER- CHANGES PER CELL
1 A	Vacuum	Vacuum	$0.12 \pm 0.01$
1 B	Vacuum	Oxygen (within 3 sec- 1500 mm of Hg)	$0.09 \pm 0.01$
2 A	Oxygen (1500 mm of Hg)	Oxygen (1500 mm of Hg)	$0.70 \pm 0.07$
2 B	Oxygen (1500 mm of Hg)	Vacuum (within 25 sec)	$0.72 \pm 0.06$
3 A	{ 1st 30 sec: vacuum 2nd 30 sec: oxygen	Vacuum	$0.32 \pm 0.02$
3 B	{ 1st 45 sec: vacuum last 15 sec: oxygen	Vacuum	$0.22 \pm 0.02$
4	1st 30 sec: oxygen 2nd 30 sec: evacuation (to 1 mm of Hg within 25 sec)	Oxygen	$0.61 \pm 0.03$

*Tradescantia*, as well as somewhat similar ones with *Vicia* root tips (Read, '52) permit the following conclusions to be drawn: (1) to be effective, oxygen must be present during the actual X-ray exposures; (2) pretreatment with oxygen does not sensitize chromosomes to subsequent X-ray breakage, as in the case of infrared treatment, for example; (3) the addition of oxygen after an X-ray exposure has no effect on

aberration yield. This last type of evidence is especially important since it indicates that, if breakage occurs in the absence of oxygen, the subsequent presence of this substance during the period when the recombination of broken ends is occurring has no effect. It appears reasonable to conclude that oxygen itself does not affect the recombination process.

It follows, then, that oxygen plus X radiation must be present simultaneously for aberration frequencies to be influenced. On this basis, the most probable explanation would appear to be that some type of reaction occurs when both oxygen and X radiation are present (a reaction which does not occur in the absence of oxygen) to produce a substance or substances which increase the aberration yield. Other explanations of this effect are, of course, possible. It can be argued that oxygen might effect the metabolism of a cell and thus alter its radiosensitivity. It is even conceivable that the presence of oxygen might modify the chromosomes themselves in some fashion to make them more radiosensitive. That explanations of this type are unlikely is indicated by the essential absence of an oxygen effect with  $\alpha$  radiation, as contrasted with X radiation (Thoday and Read, '49).

On the assumption that some substance is involved, the question then arises, and is the one which will be of principal concern in this discussion: Does such a substance cause an increased aberration frequency by increasing the initial breakage of chromosomes or by influencing the recombination of broken ends, as for example, by favoring new reunions over restitution. Initially, at least, the simplest hypothesis appeared to be one which visualized the oxygen effect as resulting from increased chromosome breakage. In particular, this hypothesis seemed a reasonable one in view of the increasing evidence that indirect effects of radiation, mediated by products produced in irradiated water, are involved in, for example, the inactivation of enzymes and other large molecules in aqueous systems (see Dale, '47). Recently, however, evidence has been presented from experiments with a number of organisms, *Zea* (Schwartz, '52), *Drosophila*



(Baker and Von Halle, '53), *Vicia* (Wolff, '54), and *Tradescantia* (Swanson and Schwartz, '53), which has been interpreted as indicating that the oxygen effect is on the recombination rather than on the breakage mechanism. In certain other experiments, however, e.g., with *Habrobracon* (Whiting, '53), and *Zea* (Konzak, '54), evidence is interpreted as supporting the breakage hypothesis.

The most extensive evidence in *Tradescantia* which has been interpreted in terms of an effect of oxygen on recombination rather than on breakage comes from the recent experiments of Swanson and Schwartz ('53) dealing with the ratios of various chromatid aberration types induced by X rays in air and in nitrogen. Their results indicate that the air/nitrogen ratios for the three types of chromatid aberrations studied — chromatid breaks, isochromatid breaks, and chromatid exchanges — are not identical and vary to some extent with the stage of prophase during which the comparisons are made. On the assumption that the ratios of all these types should be essentially identical if differential breakage is involved, the conclusion is drawn that oxygen must be affecting the recombination rather than the breakage mechanism. The assumption just mentioned appears to be invalid for at least two reasons. It is known, as in fact these authors mention, that the relation between aberration frequency and X-ray dose is not the same for all three aberration types. Isochromatid aberrations vary approximately as the 1.5 power of the dose (Kirby-Smith and Daniels, '53) and exchanges as the square of the dose, at least at high radiation intensities (Sax, '41). Reliable data for chromatid breaks are difficult to obtain, but these appear to have an approximately linear relation with dose. Furthermore, aside from these dosage-relation differences, there is no unequivocal evidence that an effect of oxygen, even if by way of the breakage mechanism, would necessarily be identical in magnitude for all three types of aberrations and all stages of the nuclear cycle, since, especially in the contrasting cases of chromatid and isochromatid types, the aberrations arise by quite different mechanisms.



Chromatid deletions result following breakage of single chromatids in those instances where restitution does not occur (and most breaks apparently reconstitute in the air as well as in nitrogen), whereas isochromatid aberrations result from the simultaneous breakage of two chromatids at the same locus. If, for example, oxygen makes a greater linear portion of an electron track effective in breakage as has been suggested by Read ('51), such a result might well have a disproportionate effect on isochromatid as compared with chromatid yields.

In this connection, it is well to recall that similar comparisons of the ratios of various chromatid aberration types were made several years ago when the first comparative studies of fast neutron and X-ray effects were made. In these comparisons, similar differences in fast neutron/X-ray ratios were found, with chromatid breaks giving the smallest, isochromatids an intermediate, and exchanges the largest values (Thoday, '42).

These results have, however, been interpreted on the basis of differential breakage, rather than as an effect of neutrons on recombination. The possibility was in fact considered that neutrons might differ from X rays in their effect on the rejoining process, but it was concluded by Thoday ('42) that "No effect of neutrons on the rejoining process could explain the increase in the number of both permanent breaks and recombination aberrations which occurs." In addition, the percentage of incomplete chromatid interchanges produced by these two types of radiation was found to be the same. Thus the general conclusion was reached that "the results cannot be explained by any differential effect of neutrons and X rays on the rejoining process and that the differences observed are due entirely to the differences in the number and distribution of primary breakage."

Preliminary observations (Giles, unpublished) of the frequencies of incomplete chromatid reunion following irradiation in the presence and absence of oxygen also indicate no

marked differences arising from exposures under these two different conditions.

It may further be noted that invoking differential recombination as contrasted with breakage to explain the different ratios of chromatid aberration types does not appear to permit a simple explanation for the results, as is evident from the various subsidiary assumptions considered necessary by Swanson and Schwartz ('53) in attempting to interpret the behavior of the chromatid deletion category.

Additional evidence in respect to the oxygen effect has been sought in experiments concerned with the average restitution time of broken ends produced in the presence and absence of oxygen. If the effect of oxygen is on recombination rather than on breakage, it might well be anticipated that this would arise from a modification of the average time of restitution of broken ends produced under these two conditions, with, for example, more rapid restitution occurring with broken ends produced in nitrogen as compared with those produced in oxygen. This possibility has been investigated for chromatid interchanges produced in *Tradescantia* microspores in experiments in which exposures were performed in nitrogen and in oxygen at a series of different X-ray intensities. The results (Riley, Giles, and Beatty, '52) indicate a similar effect of decreasing intensities on aberration yield in the two exposure series, and can be taken to indicate that restitution times are essentially the same in nitrogen and oxygen. The objection has been raised (Swanson and Schwartz, '53) that these exposures were performed at dosages giving equivalent aberration yields in the two gases rather than at equivalent physical doses. This was done on the expectation that it would make more detectable any difference in restitution time, especially if that in nitrogen proved to be more rapid. It is known, for example, that in exposures in air, a lower dose level reduces the intensity effect (Sax, '41), and it was felt that this might bias the results in nitrogen to indicate less, rather than more, rapid restitution. In any event, it is evident that additional experiments involving both intensity

and fractionation effects are desirable. This is especially true since experiments have been reported by Wolff ('54) using *Vicia faba* root tips which appear to indicate that the protective action of chemicals such as BAL against X-ray-induced chromosome aberrations arises from an effect on the restitution time of broken ends in this organism. More recent experiments (Wolff and Atwood, '54) have, however, shown that other interpretations of these results are possible.

Further general evidence concerning chromosome aberration production which appears difficult to reconcile with the recombination hypothesis may be cited. Several investigations have established that there is a reciprocal relation between the specific ionization of a given radiation and the oxygen effect. With  $\alpha$  particles, as shown by Thoday and Read ('49), there is little or no oxygen effect, whereas with fast neutrons an intermediate effect occurs (Giles, Beatty, and Riley, '52; Gray *et al.*, '53). Since recombination as well as breakage is involved in the production of aberrations with  $\alpha$  particles and fast neutrons as well as with X rays, it is not clear why irradiation in nitrogen should not result in more restitution and hence fewer aberrations with these radiations, as is postulated to occur with X irradiation. On the view that differential breakage is involved, however, a reasonable hypothesis can be developed to explain these differences in the oxygen effect with different radiations, based on differences in the spatial distribution and recombination of radicals. On this view, with X rays oxygen serves essentially to mobilize H atoms to become effective agents (possibly as  $\text{HO}_2$  or  $\text{H}_2\text{O}_2$ ) in producing initial biological damage (Giles *et al.*, '52; Gray, '53). It might perhaps be argued that such substances in the case of X rays could affect the recombination rather than the breakage mechanism. It is, however, difficult to imagine how such chemically similar substances as  $\text{HO}_2$  and OH radicals, and possibly  $\text{H}_2\text{O}_2$ , could in the case of densely ionizing particles act to produce breaks and in the case of X rays be effective only through influencing recom-

ination differentially so as to prevent restitution and further new reunions. Or if the position is taken that the effect with densely ionizing particles, as well as that with X rays in the absence of oxygen, is direct as far as breakage is concerned, it still remains difficult to see why OH radicals and  $H_2O_2$ , which must arise during irradiation with  $\alpha$  particles and neutrons, should not have an effect on recombination similar to that of  $HO_2$  radicals formed during X irradiation in oxygen. There is, however, some evidence (see Alexander *et al.*, '54) that qualitatively different chemical reactions may be produced by different types of radicals and related substances. Hence, possible explanations based on qualitatively different effects of various types of radiations in the presence and absence of oxygen cannot be completely excluded.

Additional evidence against the recombination hypothesis comes from the observations of Conger and Fairchild ('52) that in some plant cells, such as *Tradescantia* pollen, exposure to oxygen alone can result in a high frequency of chromosome aberrations. The simplest hypothesis to explain these results appears to be that, under appropriate conditions, oxygen without radiation can result in the formation of intracellular substances effective in producing chromosome breakage (see Gerschman *et al.*, '54). It is extremely difficult to reconcile these findings with the view that the oxygen effect is on recombination, since an exceedingly high rate of spontaneous breakage would be required if oxygen is effective *only* in modifying recombination so as to prevent restitution and enhance new reunions. Furthermore, the experiments discussed earlier on the postirradiation exposure of microspores to oxygen indicate that oxygen itself is ineffective in modifying the recombination process, at least with X-ray-induced broken ends.

There is one further external factor, namely, temperature, whose effect on X-ray-induced aberration frequencies should be considered at this time, since any interpretation of the mechanism of aberration production and of the oxygen effect must account for temperature effects. The considerable



amount of newly available experimental data relating to the effect of temperature on aberration production by X rays will not be discussed in detail here. Only the major results with *Tradescantia* (Giles, Beatty, and Riley, '52; Giles, '52b, and unpublished) will be summarized briefly as follows: (1) The increased yield of aberrations at low temperatures (around 1°C.) first described by Sax and Enzmann ('39) does not result exclusively from the increased solubility of oxygen at these temperatures. When oxygen is present, there is a marked effect of temperature itself in increasing aberration frequencies at low as compared with high temperatures. (2) In the absence of oxygen, the effect of temperature on aberration yields is the reverse of that found when oxygen is present, more aberrations being present at high as contrasted with low temperatures. (3) Rapid postirradiation changes in temperature following X-ray exposures in either oxygen or nitrogen apparently do not affect aberration frequencies. The temperature at the time of irradiation is the important factor.

An interpretation of all these temperature effects is by no means evident at this time. It is apparent, however, that the oxygen/nitrogen aberration ratio will depend markedly on the temperature at which the irradiation is performed. Initially, the reciprocal relation in temperature effects suggested that qualitatively different types of breaks, with respect to their behavior in recombination, are being produced in the presence and absence of oxygen, and this may indeed be true. However, the apparent lack of a postirradiation influence of temperature on recombination can be taken as evidence against this idea, and serves to emphasize once more that, as is the case with oxygen, conditions during irradiation, when breakage is occurring, rather than after irradiation, when most of the recombination of broken ends is taking place, are of major importance, at least in *Tradescantia* microspores. It may further be noted that temperature is known (Bonet-Maury and Lefort, '48) to have an effect on the yield of radicals and related products produced in ir-



radiated water, and potentially responsible for chromosome breakage.

These observations of temperature effects, however, clearly do not at present permit an unequivocal choice to be made between the alternative hypothesis we have been discussing. They do make it apparent that the mechanism of aberration production is even more complex than was originally supposed.

Before concluding, it seems pertinent to make some brief general observations concerning the oxygen effect on radiation damage to biological structures other than chromosomes. The existence of an oxygen effect or of a protective effect of certain chemicals (which is probably either directly or indirectly related to the oxygen effect) has now been demonstrated in a very large number of very different biological systems (see Patt, '53). These effects involve, for example, cell damage occurring in somatic plant cells, plant microspores, mature sperm of insects, insect larval cells, and other cells. In mammals, chemical protection has been observed against such effects as lethality, lymphopenia, granulocytopenia, splenic involution, epilation, and lenticular opacities (Brues and Patt, '53). Furthermore, the magnitude of the oxygen effect as well as the shapes of the curves relating increasing oxygen tension to the biological effect of X rays are remarkably similar in many of these materials (Gray *et al.*, '53). In addition, it is clear that at least in most cases, oxygen or chemicals are effective only when they are present during and not after the irradiation. These similarities may be taken to imply, as Gray *et al.* ('53) and Patt ('53) have indicated, a common feature either in the chemical changes which follow directly from the absorption of radiation energy in the cell or in the biochemical pathway by which all these lesions develop. It would appear to be much simpler to interpret such results on the basis of increased initial damage by substances such as active radicals to vulnerable biological molecules or structures rather than to an effect of such radicals in modifying recovery processes (wherever recovery occurs),

since these recovery processes must be quite various in the very diverse systems affected. In particular, they are presumably quite different from the rather special type of situation which obtains for so-called recovery in the case of induced chromosome aberrations.

In closing, it should be noted that the predilection to explain all related experimental observations on the basis of a single hypothesis is, of course, always a considerable temptation. It is, moreover, generally defensible on the basis of the principle of William of Occam. It may well be, however, that in this particular instance an attempt is being made to utilize a razor with too keen an edge. It is, for example, quite possible that in the specific situation under discussion, both differential breakage and differential recombination of broken ends may be involved in the reactions leading to aberration production, or that the oxygen effect may be operating by different mechanisms in different organisms. In any event, it is clear that experiments dealing with the oxygen effect, whatever their eventual interpretation, are certain in the long run to contribute to a better understanding of the basic mechanisms by which ionizing radiations produce chromosomal aberrations, as well as other biological effects.

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# THE OXYGEN EFFECT AND CHROMOSOME BREAKAGE<sup>1, 2, 3</sup>

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## ONE FIGURE

The early data derived from studies dealing with the effects of ionizing radiations on the production of chromosome aberrations were readily understandable from a purely physical standpoint, and the concepts concerned with the interpretation of these data have been ably summarized by Lea ('46). Emphasis is shifting from the physical to the chemical point of view, however, and this tendency to think of cellular damage in terms of radiation chemistry is forcing a revision of ideas (Gray, '53; Thoday, '53). It is no longer possible to consider that the breakage of chromosomes and the later restitution and recombination of broken ends are the only events leading to the production of aberrations, for studies which have demonstrated that the final frequency of aberrations is subject to wide modifications by a number of supplementary physical and chemical means make it difficult, indeed, to avoid the conclusion that ionizing radiations produce a variety of primary changes in the chromosome. The nature of these initial changes has not yet been well elucidated, but at least for purposes of convenience the changes can be considered sites of damage which, by modifying the cellular environment,

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<sup>2</sup> A brief version of this paper was presented to and published by the Radiobiology Symposium, Liège, Belgium, 1954; figure 1 will also be presented by K. G. Lüning (1954) in *Hereditas*.

<sup>3</sup> Paper read at this meeting by Dr. H. Bentley Glass.



may be repaired or amplified. Experimentally, the chemical mutagenic studies of Auerbach ('51), the infrared-X-ray results of Swanson and Yost ('51), and the work of Alper on irradiated bacteriophage (quoted by Gray, '53) force a consideration if not an actual adoption of this point of view. The theoretical aspects of this approach have been discussed by McElroy and Swanson ('51); Thoday ('53) and Gray ('53) have dealt with the problem in some detail. Gray ('53, p. 614) has stated that "the main features of the biological experiments make very good sense when viewed from the standpoint of radiation chemistry," an hypothesis, however, which supplements rather than contradicts the ideas developed by Lea to show that chromosome breaks are produced by the passage of single ionizing particles through or near the chromosome. These considerations take on a particular pertinence when applied to the problem of the effect of oxygen on breakage by ionizing radiations, but previous studies make it almost certain that oxygen, infrared, and ultraviolet, in modifying the rate of breakage by ionizing radiations, act through quite dissimilar pathways. It must be assumed, therefore, that the primary events induced by ionizing radiations differ not only in degree but in kind, and that their nature will determine what extraneous agent will bring about repair or amplification of the damage. For convenience, one can consequently speak of "direct" and "indirect" energy transfer to the chromosome, with "direct" energy being that contributed by the ionizing particle itself, and "indirect" energy that mediated by extraneous agents and, presumably at least, one step removed from the initial ionization events.

In this instance the discussion will be confined to the possible levels at which oxygen can affect chromosome breakage, and a scheme, arising out of a collaboration with Dr. K. G. Lünig of the University of Stockholm and included here with his kind permission, will be presented which takes into account the hypothesis that ionizing radiations produce a variety of primary events in the chromosome, and that these events are subject to modification.

## EARLIER STUDIES

The work of Thoday and Read ('47, '49) and of Giles and his coworkers (Giles, '52; Giles, Beatty, and Riley, '52; Riley, Giles, and Beatty, '52) have established beyond reasonable doubt that oxygen is part of a reactive system which can modify the frequency of aberrations produced by ionizing radiations. This system owes its origin apparently to the interaction of dissolved molecular oxygen with the products of ionized water (Gray, '53), a conclusion reinforced by the fact that during irradiation oxygen can be removed from the cell by evacuation or by replacement with hydrogen, argon, helium, or nitrogen, and the effect of such induced anoxias on breakage is essentially similar. Furthermore, posttreatment with oxygen is without effect (Thoday and Read, '49; Giles and Riley, '49).

Secondly, it is now quite clear that the degree of decreases resulting from irradiation during anoxia is not the same for all types of aberrations even when the quality of radiation is kept constant. Earlier studies seemed to suggest that, for a variety of effects, the ratio of maximum radiosensitivity to anoxia radiosensitivity was roughly the same, viz., about 2.5:1 (Gray *et al.*, '53), but new evidence demands either a modification of this concept or a reevaluation of the steps leading to chromosome breakage. This was first indicated by the data of Riley, Giles, and Beatty ('52), who demonstrated that chromatid deletions were less affected by the absence of oxygen than isochromatid deletions and chromatid exchanges. If the reduction in frequency of aberrations is expressed as an air/nitrogen ratio, then that for chromatid deletions was about 1.4 as compared to 2.6 for isochromatid deletions and exchanges. These differential relations of radiosensitivity have been confirmed (Swanson and Schwartz, '53), and, in addition, it was possible to demonstrate that the air/nitrogen ratios for the three types of chromatid aberrations do not remain constant throughout the prophase period of the mitotic cycle. Thus, for the 8-, 12-, and 24-hour periods after exposure to X rays, the air/nitrogen ratios for chromatid

deletions were 1.1, 1.1, and 1.0; for isochromatid deletions 2.8, 2.6, and 2.3; and for exchanges 2.9, 2.4, and 2.0, respectively. Essentially similar results have been obtained for various genetic effects in *Drosophila* (Hollaender, Baker, and Anderson, '51; Baker and Edington, '52; Baker and Von Halle, '53; Lüning, '54). It cannot therefore be expected that a general reduction in radiosensitivity will be achieved by irradiation under conditions of anoxia, but that each effect has its own

TABLE 1

*Chromatid aberrations produced in Tradescantia by four qualities of radiation in air and in nitrogen. 150 r at 8.9 r/minute*

RADIATION	ATMOSPHERE	NO. OF ABERRATIONS PER 100 CELLS		
		Chromatid deletions	Isochromatid deletions	Exchanges
X rays — 50 kvp, unfiltered	Air	49.3	84.6	35.3
	Nitrogen	98.2	15.0	9.5
	Air-nitrogen	0.50	5.6	1.9
X rays — 100 kvp, 1 mm of Al	Air	66.0	62.5	30.5
	Nitrogen	79.3	14.0	7.3
	Air-nitrogen	0.83	3.9	2.0
X rays — 250 kvp, 4 mm of Cu	Air	78.0	44.0	28.0
	Nitrogen	63.5	16.6	6.0
	Air-nitrogen	1.25	2.7	2.3
$\gamma$ Rays — 1.1–1.3 Mev	Air	96.2	32.5	26.0
	Nitrogen	50.5	14.0	5.0
	Air-nitrogen	1.9	2.3	2.3

response which may vary with the cellular circumstances. Any hypothesis which seeks to explain the oxygen effect must, as a consequence of these data, be flexible enough to account for differential reductions. Present theories are inadequate in this respect.

Thirdly, it has been established that the influence of oxygen tension on the frequency of radiation-induced aberrations is intimately linked to the type of radiation employed. Thoday and Read ('49) have demonstrated that the oxygen effect is much less with  $\alpha$  rays than with X rays, while neutrons appear

to occupy an intermediate position (Giles, Beatty, and Riley, '52). It would be reasonable therefore to suppose that the ion density of the radiation is of primary importance in determining the magnitude of the oxygen effect; the effect, in fact, is insignificant for ion densities above 300 ions/ $\mu$  of path length (Gray, '53). Studies carried out on the microspore chromosomes of *Tradescantia* have a particular bearing on this point. Although presented in detail in another report (Swanson, '55), the data can be briefly summarized here (tables 1 and 2).

TABLE 2

*Percentage reduction in total breakage in Tradescantia as the result of exposure in the absence of oxygen*

RADIATION	DERIVED FROM TABLE 1	MISCELLANEOUS <sup>a</sup>
$\gamma$ Rays — 1.1–1.3 Mev	59	
X rays — 250 kvp	48	58
X rays — 100 kvp	43	
X rays — 50 kvp	36	
Neutrons		33
$\alpha$ Rays		0

<sup>a</sup> Reduction of 58% at 250-kvp X radiation was extracted from Riley, Giles, and Beatty ('52; table 1, at 150 r). The neutron and  $\alpha$ -ray reductions were derived from data in Giles, Beatty, and Riley ('52; table 2, at 10 n). Thoday and Read ('49) indicate that some reduction is obtained with  $\alpha$  rays when exposure is made in nitrogen.

Use has been made of three qualities of X radiation together with 1.1- to 1.3-Mev  $\gamma$  rays from the Co<sup>60</sup> facility described by Kirby-Smith and Daniels ('53). It is apparent (table 1) that in air the chromatid deletions increase in frequency as the ion density of the radiation decreases, whereas the reverse holds true for isochromatid deletions. This shift in frequency appears to be a compensatory one, for the total frequency of deletions (i.e., chromatid plus isochromatid) does not vary appreciably with quality of radiation. These data are somewhat surprising since Kirby-Smith and Daniels ('53), using pollen tube chromosomes of *Tradescantia*, demonstrated that



both types of deletions are more numerous with X rays than with 400-kv  $P^{32}$   $\beta$  rays and  $Co^{60}$   $\gamma$  rays. No compensatory shift was found by them. It may well be that the dose (150 r) and the time of fixation after irradiation (19–20 hours) employed in these experiments were such as to give the precise relations observed, but four rather extensive and essentially similar experiments yielded similar results.

The relation of chromatid exchanges to quality of radiation in air is similar to that found for isochromatid deletions, and these data are in agreement with the earlier work of Kirby-Smith and Daniels ('53).

When irradiation was carried out under conditions of anoxia, the isochromatid deletions, although greatly reduced in frequency, appeared to show no relation to quality of radiation. This, of course, leads to air/nitrogen ratios which are large with 50-kvp X rays, and which decrease progressively in value as the ion density of the radiation decreases. The chromatid deletions, on the other hand, now exhibit an opposite trend from that characteristic of the air series. They increase rather than decrease as the ion density of the radiation increases, and consequently the air/nitrogen ratios are highest in value when  $\gamma$  rays are used and lowest with 50-kvp X rays. With 50- and 100-kvp X rays, there is, in fact, an actual increase in the absolute frequency of chromatid deletions obtained in nitrogen as compared to that found in air, and the air/nitrogen ratios fall below 1 in value. The chromatid exchanges, like the isochromatid deletions, are greatly reduced, but the air/nitrogen ratios follow a trend comparable to that for chromatid deletions, as might be expected.

As pointed out in an earlier account of this work (Swanson, '55), it is believed that the differential air/nitrogen ratios for the three types of aberrations can be accounted for by assuming that there is a shift, when irradiations are carried out under anoxic conditions, of one type of aberration into another. Isochromatid deletions and chromatid exchanges each involve two broken chromatids, although they are dissimilar in that the former arises from broken sister chromatids



and the latter from broken nonsister chromatids. The repair or restitution of one of these chromatids but not of the other would transform potential isochromatid deletions and exchanges into chromatid deletions. Additional support for this belief is provided by the study of isochromatid dosage curves in air and in nitrogen. These were compared at 50- and 250-kvp X rays (the former unfiltered and the latter filtered through 4 mm of Cu), and in air the powers for the two curves were 1.67 and 1.50, respectively. These values are higher than expected, but they are in essential agreement with similar data of Kirby-Smith and Daniels ('53) and Thoday ('53). Under anoxic conditions, and if a process of repair is favored by these circumstances, it would be expected, because of the time element involved, that not only would a reduction in frequency of isochromatid deletions be found but also that a straightening of the curves would result through the preferential elimination of the two-hit as compared to the one-hit category. The data obtained from irradiations in nitrogen were in accord with this hypothesis since the corresponding curves had powers of 1.08 and 1.27.

Such a change in the shape of the dosage curves would not be expected for aberrations produced in chromosomes which were single-stranded at the time of irradiation (i.e., rings and dicentrics), or for aberrations in double-stranded chromosomes which were exclusively one-hit (chromatid deletions) or two-hit (chromatid exchanges) in origin. Anoxia would simply behave as a dose-reduction phenomenon without the preferential elimination of a particular type of aberration such as the two-hit isochromatid deletion. That these expectations have been realized has been demonstrated by Riley, Giles, and Beatty ('52) and confirmed by us.

There remains another possible explanation for the chromatid-isochromatid deletion shift observed in the air versus the nitrogen series (table 1). This possibility, suggested by Dr. O. C. A. Scott of England (personal communication) is concerned with the geometry of the terminal portion of the electron paths. As Gray ('53) has indicated, no oxygen effect

is observed when the ion density is above 300 ions/ $\mu$  of path length. The terminal portion of every electron track is densely ionizing, and a density of 300 ions/ $\mu$  will be passed when the energy of the electron drops below 0.75 ekv (Lea, '46, pp. 24-25). The further penetration of such an electron is, however, only about 0.035  $\mu$  in tissue. It would be expected, therefore, that the terminal 0.035  $\mu$  of each track would show no oxygen ionizing, and a density of 300 ions/ $\mu$  will be passed when the estimated diameter (0.1  $\mu$ ) of a *Tradescantia* chromatid. Lea (p. 275) estimates that the *effective* "tail" length is 0.28  $\mu$ , and this corresponds to an electron energy of 2.8 ekv having an initial rate of 100 primary ions/ $\mu$ . Consequently, part of the tail should be less effective in nitrogen, possibly by a factor of 2 to 3. When *Tradescantia* chromosomes are irradiated in nitrogen, and an electron has sufficient energy to traverse both chromatids, the probability of the first chromatid being broken will be less than that for the second chromatid, and a shift from isochromatid to chromatid deletions would perhaps be expected. The argument would still hold even if the entire length of the effective tail showed an oxygen effect, since the last 0.1  $\mu$  of path length would have a higher probability of breaking a chromatid than the first 0.1  $\mu$ .

This possibility, which, in effect, is based on the consideration that anoxia alters the effective geometry of the ion tracks, has much in its favor to commend it, and while the author is not fully capable of appraising it in all its physical aspects, it is believed to be more applicable to softer radiations than those employed in these experiments. Furthermore, it is somewhat difficult to see how this hypothesis can account for the drop in value of the power for the isochromatid dosage curves in nitrogen as opposed to those in air. In terms of two-hit aberrations, the system described would function simply as a dose-reduction phenomenon, but it is unlikely to create a situation which would preferentially reduce the two-hit isochromatid deletions as compared to the one-hit type in nitrogen.

## PREVIOUS HYPOTHESES

Two hypotheses, diametrically opposed to each other, have been advanced to account for the effects of oxygen on the production of aberrations by ionizing radiations. Neither hypothesis in its present form can satisfactorily encompass the available data, largely, it is believed, because breakage and restitution have been viewed in rather unequivocal terms in that they are considered to be the only possible steps subject to experimental modification by variation in oxygen tension. Operationally, breakage and restitution are easily distinguished from each other, but if the point of view of Gray ('53) is adopted that a variety of primary events are induced by ionizing radiations, then additional steps, subject to modification, are possible between the introduction of radiation into the cell and the final realization of completed aberrations.

The restitution hypothesis, advanced by Schwartz ('52) and supported by Baker and Von Halle ('53) and Swanson and Schwartz ('53), proposes that anoxia does not affect breakage, but instead favors the joinability of broken ends, perhaps because of a quantitative difference in the nature of the ends broken in nitrogen as compared to air. The data in table 1 can be easily explained by the restitution hypothesis if it is assumed that a process of partial restitution can transform potential isochromatid deletions and exchanges into chromatid deletions. Difficulty is encountered, however, when the neutron and  $\alpha$ -ray data are considered in these terms. As table 2 indicates, there is a definite relation of oxygen effect to quality of radiation, but it must be recognized that the chromatid deletion/isochromatid deletion ratio is generally lower with particulate radiations than it is with X or  $\gamma$  rays. Consequently, if our hypothesis that isochromatid deletions and exchanges can be transformed under anoxia into chromatid deletions through a process of partial restitution is correct, it would be expected that the air/nitrogen ratios for chromatid deletions and exchanges would be lower in value, and those for isochromatid deletions higher, than the corresponding values found with X rays. The studies of Thoday and Read

('49) with  $\alpha$  rays and of those of Giles, Beatty, and Riley ('52) with neutrons reveal that these expectations are not realized. It must be assumed therefore either that the ends of chromosomes broken by particulate radiations are qualitatively different in their rejoinability as compared to those broken by X rays, or that restitution as defined by Schwartz is not an important process in *Tradescantia* as regards to oxygen effect. The latter appears to be the more likely possibility.

The breakage hypothesis of Giles ('52; Riley, Giles, and Beatty, '52) merely specifies that the breakage mechanism is influenced by the oxygen tension of the cell. Although it is considered to be the more acceptable hypothesis, amplification of the manner by which oxygen acts is now both possible and desirable. If breakage alone is involved, it is difficult to imagine how an increase in any type of aberration can occur as the result of a reduction of breakage under conditions of anoxia. The suggestion of Scott, presented earlier, that the chromatid/isochromatid shift as a function of an altered geometry of electron tracks, must of course remain as a possibility, but it is felt that the scheme presented in the following section satisfactorily explains the available data and is also consistent with physical facts.

#### A NEW INTERPRETATION

With the collaboration of Dr. Lüning, the scheme in figure 1 was conceived of as a diagrammatic representation of the possible ways in which oxygen could influence the frequency of aberrations produced by ionizing radiations. It is, in a sense, a modification and a simplification of the scheme presented by Thoday ('53), and departs from it principally by the inclusion, in phase II, of an element which we have chosen to call a "spectrum of chromosomal damage." The impelling reasons for this concept are several. First, and as stated in the introduction, there is good evidence for the belief that a variety of primary events can be induced by ionizing radiations, events which are capable of repair or of further



development by physical or chemical means. Our "spectrum" would therefore include what Thoday ('53) has called "potential" breaks. Second, this concept was considered necessary because it is quite inconceivable that two radiations as different as  $\gamma$  and  $\alpha$  rays would give rise to the same

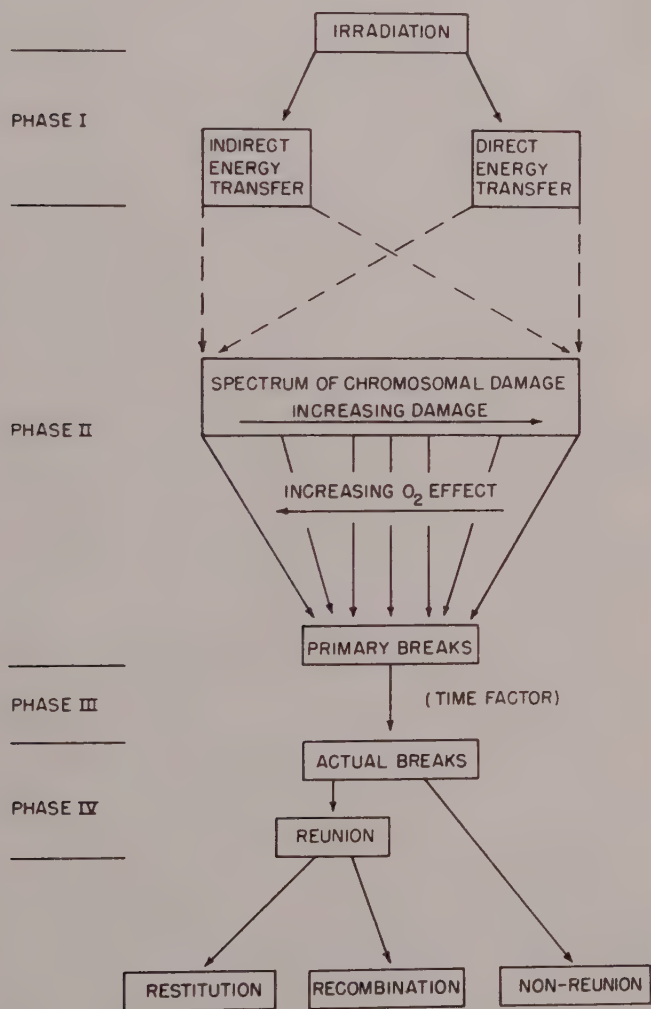


Fig. 1 A diagrammatic scheme of the possible levels at which oxygen could influence the effects of ionizing radiations (from Lünig, '54).



spectrum of chromosomal damage. Thus, even assuming that the number of damaged sites produced by these two radiations were equal in number, it is further assumed that the extent of damage per site induced by  $\gamma$  rays would be less, and consequently lie to the left of the spectrum, and the damage by  $\alpha$  rays to the extreme right. The arrow within the box indicates therefore the direction of increasing damage, and a shift to the right or left would be a function principally of the ion density of the impinging radiation.

As to the steps in figure 1, phase I includes the initial physical and chemical events induced by radiation, and for convenience the direct energy available for transfer to the chromatin strands can be considered that resulting immediately from the ionizing particle whereas indirect energy would be available for transfer, presumably from ionized water, only through an additional chemical reaction. The total ionization would be independent of oxygen tension, but the utilization of the indirect fraction would be dependent on the oxygen tension, or on other physiological circumstances within the cell which could interfere with the transfer of energy to the chromosome.

In phase II, oxygen through the medium of indirect energy transfer could act in several ways. It could contribute itself to the spectrum of damage, it could enhance the damage already created by direct energy transfer, or presumably both actions could be involved. Anoxia therefore could exert its effect either by decreasing the damage, or, through failure of enhancement, permit repair of damage to proceed. Experimentally, a distinction cannot be made in the present material, but preference for the latter possibility is indicated by the arrow (increasing oxygen effect) being placed below the spectrum of damage. In any event, it is presumed that oxygen would exert its most pronounced effects at the left end of the spectrum, it being of greater relative importance in the transformation of slight damage to the level of primary breaks than for the more extensive damage at the right end. If this is true, then the relations indicated in table 2 are as expected.

Phases I and II include steps which are part of the breakage mechanism. In phase III, primary breaks differ from actual breaks only by a time factor. In *Tradescantia* the time element would be of brief duration, and of negligible importance when the intensity of radiation is high, but in *Drosophila* sperm, where apparently the time element is of greater duration, oxygen could conceivably play a role. Phase IV is believed to be unimportant in *Tradescantia* as regards the oxygen effect. It is at this level that Schwartz ('52) would have oxygen exerting its principal effect, but reasons have been given for considering this an unlikely possibility.

To what extent the scheme presented reflects the actual events taking place in the cell is, of course, problematical. In the light of available data it appears reasonable. Future studies, however, will test the validity of the several assumptions made.

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# EVIDENCE ON THE MECHANISM OF THE OXYGEN EFFECT BY USE OF A RING CHROMOSOME<sup>1</sup>

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## THREE FIGURES

A restatement of the problem under discussion is unnecessary since the previous speakers have clearly explained the ramifications of the breakage and reunion hypotheses of the oxygen effect on irradiated chromosomes. The work to be discussed represents an attempt to get an unequivocal answer to the question at hand. Theoretically, either of two situations could provide the necessary information. In the first place, if the condition of a chromosome at the time of irradiation, and for the subsequent period of rejoining, were such that no reunion of induced breaks were allowed, fewer broken chromosomes should be observed upon irradiation in low oxygen concentrations if the oxygen were acting on the initial number of breaks produced. Alternatively, if the reunion hypothesis were valid, no oxygen effect should be observed. Secondly, if the converse situation were provided where all breaks of a given chromosome rejoin, no effect on intrachromosomal rearrangements of this chromosome should be seen if oxygen were affecting the reunion process. We selected the latter situation, which is more amenable to testing, since the  $X^{c1}$  ring of *Drosophila melanogaster* provides the necessary requirements.

Figure 1 depicts the events which can take place when a single break is primarily induced in a ring chromosome. It

<sup>1</sup> This work was performed under contract No. W-7405-eng-26 for the Atomic Energy Commission.

should be noted that it is inconsequential to the following argument whether the ring is considered as being divided into chromatids in the mature sperm — Cooper ('49) has presented evidence that this is true — or considered as single, since a twist in the single thread following breakage accomplishes the same end result as an exchange between sister chromatids; i.e., the formation of a dicentric double-ring chromosome. Catcheside and Lea ('45) showed that a two-armed X chromosome is never recovered from irradiated sperm containing a

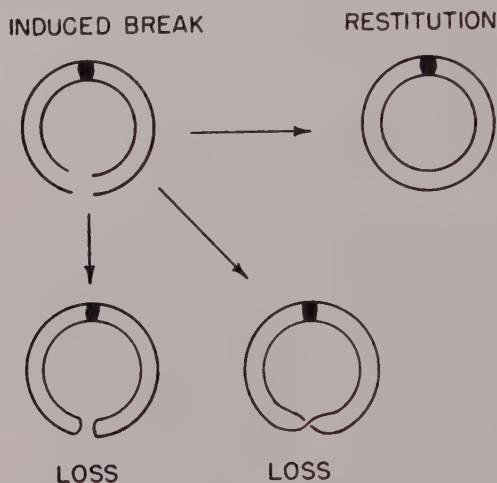


Fig. 1 Manner in which a break may rejoin in a ring chromosome.

ring X; obviously, then, the broken ends must join, and this could be accomplished by the three ways shown in figure 1. Two of these ways lead to elimination of the X chromosome, an event which can be detected genetically by the following scheme: Males homozygous for the second chromosome mutant, *brown*, and bearing the  $X^{c,y}$  ring and the Y chromosome of Dempster which contains an insertion of  $bw^+$  in its long arm, were irradiated and mated individually to  $y\ v; bw$  females. The normal  $F_1$  males from this cross will have vermilion eyes whereas the X-O males, which arise from female zygotes that lose the ring chromosome during the



first cleavage division (presumably by the types of rejoining previously shown), will have white eyes, the phenotypic expression of the vermilion-brown combination. Similarly, in the crosses where the *scute*<sup>8</sup>·Y (containing the wild-type allele of *yellow* in Y<sup>+</sup>) was used, the X-O males would be yellow. It is obvious that loss of the X<sup>c</sup> chromosome will have two measurable effects: (1) a shift in the sex ratio to favor males since some of the female zygotes are being converted into males and (2) the appearance of X-O males. It should be noted that loss of the marker on the Y chromosome will produce a male which is phenotypically identical to one produced by loss of the ring; however, this process will have no effect on the sex ratio.

The experiments were carried out in the following manner. Males bearing the ring chromosome and the marked Y were irradiated with 250-kvp X rays (30 ma, 2-mm Al filter giving a dose rate of about 260 r/min) in a continuous flow of either air or nitrogen and immediately after treatment were allowed to mate individually with the *y v; bw* females for 24 hours. After this period each male was mated to a new female for another 24 hours, following which time he was removed; the female remained in the culture vial to produce offspring. By this procedure two samples of sperm which were mature at the time of irradiation were procured.

The data obtained when the Y chromosome was marked with the *bw*<sup>+</sup> insertion are shown in table 1 and figure 2. Since no difference was found in the sex ratio or the frequency of X-O males between the two samples of sperm tested (see table 1 for single exception), these data have been combined. Certain features of these curves should be noted. In the first place, there is no difference in the frequency of X-O males between the air and nitrogen series at the low dosage, 1000 r. Even the difference at 4000 r is not significant, although a real difference might be expected from considerations to be discussed later. Likewise, oxygen has no effect on the sex ratio at the low dose; but at the higher doses, significantly more males are produced when irradiation is carried out in

TABLE 1

*Relation between dosage and events implying loss of X<sup>c</sup>*

X-RAY DOSE (r)	GAS	X <sup>c</sup> /Y: bw <sup>+</sup>			X <sup>c</sup> /Y: y <sup>+</sup>			
		SPERM BATCHES 1 AND 2			SPERM BATCH 1		SPERM BATCH 2	
		Total flies	Male freq.	Freq. of X-O	Total flies	Male freq.	Total flies	Freq. of X-O
Control	Air	13,345	0.500	0.0051	4265	0.539	3055	0.0052
	N <sub>2</sub>	10,621	0.502	0.0040				
1000	Air	7,833	0.551	0.0151	3721	0.589	2575	0.0136
	N <sub>2</sub>	7,462	0.545	0.0166	3282	0.575	1684	0.0202
2000	Air	4,319	0.588	0.0257				
	N <sub>2</sub>	4,253	0.579	0.0223				
3000	Air	3,970	0.598	0.0330	1006	0.635	780	0.0372
	N <sub>2</sub>	3,928	0.588	0.0305	1855	0.622	1426	0.0231
4000	Air	3,751	0.638	0.0355				
	N <sub>2</sub>	5,665	0.641 <sup>*</sup>	0.0312				

<sup>\*</sup> Sperm batch 2 was significantly higher than sperm batch 1.

air as contrasted to nitrogen. Therefore, it is apparent that, although the air and nitrogen dose curves for sex ratio are identical at low doses, they diverge at higher dose levels.

Let us now return to a consideration of the premise on which these experiments were undertaken. It was stated pre-

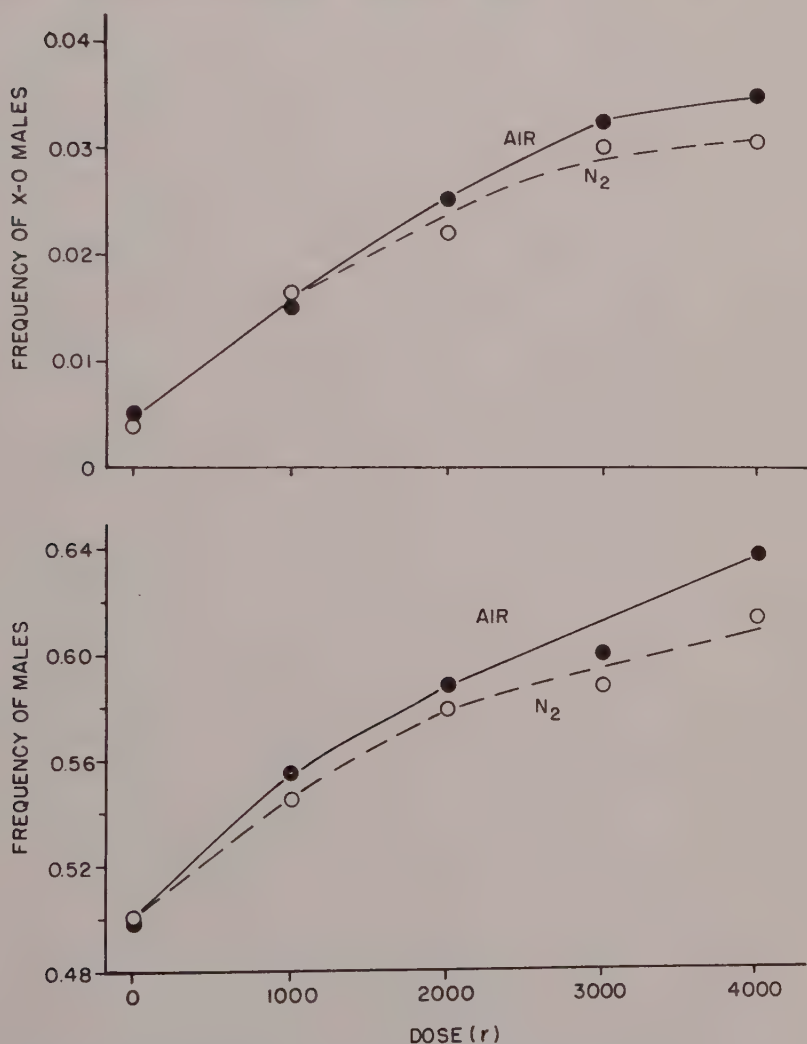


Fig. 2 Dose-effect relation in which the *bw*<sup>+</sup>-marked Y chromosome is used. Solid circles, air; open circles, nitrogen.

viously that, if the reunion hypothesis is correct, no oxygen effect would be observed on intrachromosomal rearrangements (loss of the ring chromosome) involving a chromosome in which all the broken ends are compelled to unite. However, the sex ratio is shifted not only by intrachromosomal rearrangements, which cause loss of the ring, but also by interchromosomal exchanges with the ring, all of which produce dicentric chromosomes leading to death of female zygotes. The shift in the sex ratio caused by this factor comes into play only at higher dose levels where a break in the ring is accompanied, in the same sperm, by at least one break in an autosome. On the basis of the reunion hypothesis, this process would be oxygen dependent since, if breaks produced in nitrogen are more likely to reconstitute (the only type of rejoining which takes place in mature *Drosophila* sperm), there would be fewer of these two-hit aberrations in the nitrogen as compared to the air series. This, in turn, means that the nitrogen-treated males would retain a slightly higher frequency of sperm with a break only in the ring and none in the autosomes than would be present in the sperm treated in air. If the loss of part of these remaining broken ring chromosomes by intrachromosomal recombination is oxygen independent (as would be expected on the reunion hypothesis since all breaks rejoin), a slight excess of X-O males produced in nitrogen over air would be expected. On the other hand, if oxygen were acting by increasing the initial number of breaks, a measurable effect should be observed at all doses in both the sex ratio and frequency of X-O males. The data clearly show that the latter expectation is not realized, whereas the former is in agreement with the experimentally observed facts. Therefore the breakage hypothesis appears to be invalidated.

The slight, but not statistically significant, excess of X-O males in the air series is not unexpected when one considers that loss of the *bw*<sup>+</sup> marker on the Y may occur in a few cases by breaks produced proximal to the insertion. On the reunion hypothesis, this event would be oxygen dependent and would

lead to a small oxygen effect on the frequency of X-O males, but would not affect the sex ratio. The role of this factor in influencing the frequency of X-O males is clearly seen in experiments with the *scute*<sup>s</sup>.Y chromosome where the marker,  $y^+$ , is located at or near the tip of  $Y^L$ .

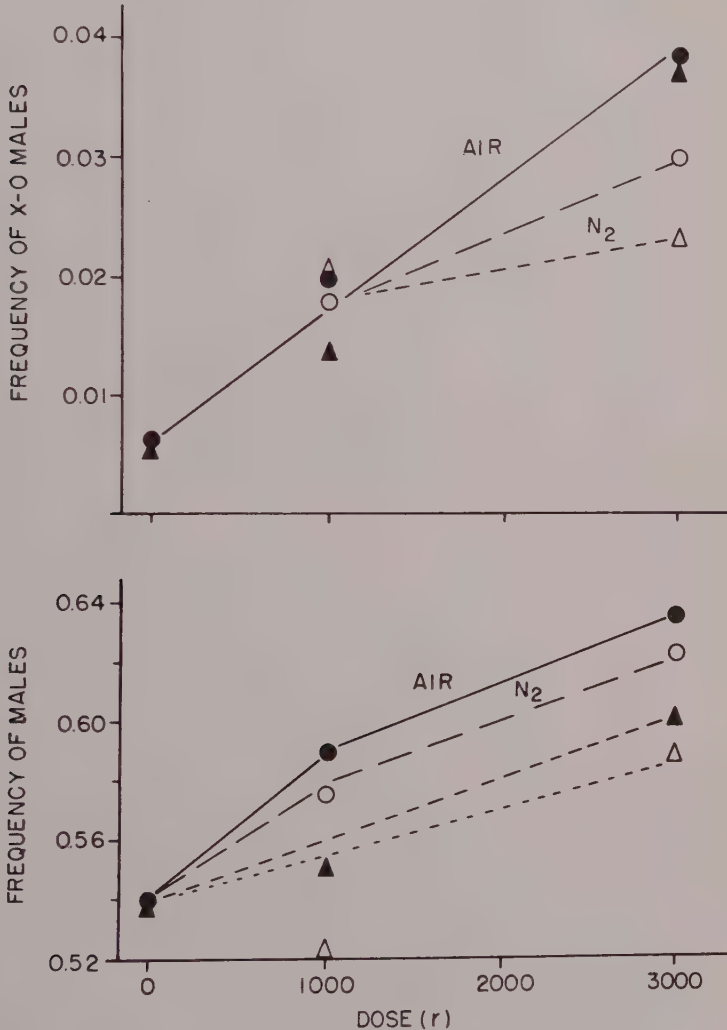


Fig. 3 Dose-effect relation in which the  $y^+$ -marked Y chromosome is used. Solid circles, air (1 sperm batch); open circles, nitrogen (sperm batch 1); solid triangles, air (sperm batch 2); open triangles, nitrogen (sperm batch 2).



The data we obtained using this particular Y are shown in table 1 and figure 3. A difference between the two sperm batches sampled was observed with both criteria of effect. Although the reason for this difference is not clear at the present time, we feel that this finding is not pertinent to the point under discussion since it is observed with treatments in either gas. The particular point of interest in these curves is the oxygen effect observed at 3000 r on the frequency of X-O males which is unaccompanied at this dose by any great shift in the sex ratio observed in the two gas series. Such an effect was not observed with the Y marked with the *bw*<sup>+</sup> insertion. However, this is just what would be expected with a Y marked out at the tip since there would be more breaks produced proximal to the marker than when the marker is interstitially located. This would lead to increased loss of the marker, thereby producing more apparent X-O males but leaving the sex ratio unaffected. Therefore, we conclude that the apparent oxygen effect with a ring chromosome observed by using the criterion of the X-O male frequency is not caused by loss of the ring but rather by loss of the *y*<sup>+</sup> marker at high doses.

Since our results (see also Schwartz, '52; Baker and Von Halle, '53) appear to favor so strongly the reunion hypothesis whereas the findings in *Tradescantia* and *Vicia* are still equivocal, it might be well to mention two features of the *Drosophila* experiments which differ from the plant studies. The chromosomes of the treated cells (mature sperm) are all at a known stage at the time of treatment since the cells are not mitotically active. This is in contrast to the plant studies where the effect is observed at a particular stage in the mitotic cycle and all cells observed in this stage at the time of fixation are assumed to have been at one stage at the time of treatment. However, differential behavior of the chromosomes during the period between treatment and fixation, in response to different treatments, could invalidate this assumption. Another unusual feature of treating *Drosophila* sperm is that restitution and sister fusion are the only types of rejoining which could

possibly take place in the mature sperm. This has been made evident by many investigators who have failed to find an intensity effect on chromosomal interchanges. Thus the process of restitution and sister fusion are isolated in time from the other types of rejoining. It remains to be seen whether these simplified conditions of the *Drosophila* experiments are responsible for the rather clear confirmation in this organism of the reunion hypothesis of oxygen action.

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## DISCUSSION OF THE LAST THREE PAPERS

### TWO FIGURES

CONGER: It is possible, in *Tradescantia*, to measure any possible effect that oxygen might have on the fusibility of the ends of the chromosomes that are broken by the radiation. I shall present the argument on the assumption that the oxygen does have an effect on the fusibility of broken ends. The data will show that it does *not* have an effect, but it is easier to present it on the assumption that it does.

The argument is this. Let us look first at one of the kinds of aberrations that Swanson talked a lot about, namely, the isochromatid aberrations which are formed in the manner shown in figure 1. A chromosome is broken by radiation; this is the initial break. The question is: What can happen to this afterward in *Tradescantia*? Unfortunately, what happens in most of the cases is that it "restitutes" or reunites in a way which you cannot detect — the restitution class, in the figure. The ends can also fuse in the other way shown, but this you cannot see either, and this is why there is a lot of argument on this point. Most of these events, or so-called primary breaks, result in restitution which the cytologists cannot see.

But the sister-union (SU) class, of course, is also a fusion of the broken ends produced by the radiation. The events that are observed cytologically are those which do not result in restitution. If, instead of fusing to form restitution, the broken ends happen to fuse in the other ways shown, the primary break is then visible as a chromosome aberration called the isochromatid type. The fact is that in the *Tradescantia* experiments the visible isochromatid aberrations are produced about 2.5 times as often in oxygen as in nitrogen.

On closer examination of these isochromatid aberrations, however, other kinds are cytologically distinguishable — the nonunion (NU) types.

The chromosome is broken as shown in figure 1. The ends may reconstitute, and in that case nothing is visible. The two ends can fuse to form the first kind of aberration; this is called sister union. The proximal end may not fuse and the distal end fuse (NU<sub>p</sub>); or the opposite of this (NU<sub>d</sub>); or, in the last case, neither end may fuse at all (NU<sub>pd</sub>).

If, in observation of these isochromatid aberrations, you distinguish which class they belong to, it is obvious that if







ISOCID BREAK (INITIAL BREAK)					
AFTER FUSION, MAY RESULT IN:	NOT OBSERVED	ISOCHROMATID ABERRATIONS (OBSERVED)			
					
CLASS	RESTITUTION	SISTER UNION (SU)	NONUNION PROXIMAL (NU <sub>p</sub> )	NONUNION DISTAL (NU <sub>d</sub> )	NONUNION PROX AND DISTAL (NU <sub>pd</sub> )
NUMBER OF FUSIONS	2	2	1	1	0

Fig. 1 Fusion fate of an isochromatid (isocid) break.

oxygen is having an effect on the fusibility of broken chromosomes — let us assume this is how oxygen does exert its effect, remembering that since we cannot see the restitution class we do not know how often it occurs and we can only work with those which we see — then it should cause a shift in the direction of the aberrations toward the right on the figure, i.e., toward those in which there is only one fusion or none at all. The data which Miss Helen Johnston and I have collected on this show that there is no difference in the frequency of these different fusion classes in oxygen or nitrogen, indicating that the fusibility of isochromatid broken ends is the same when they are irradiated in oxygen or nitrogen.



A similar sort of reasoning can be applied to another kind of aberration, the exchange type, of which Swanson has talked. These also can be analyzed for the completeness or incompleteness of the fusion in the manner shown in figure 2. It is identical to the other analysis we have just made for isochromatid aberrations, which are included on the figure so the comparison will be more apparent.

If two breaks are produced in a cell, they may fuse at random to form exchange aberrations which are visible; and these fusions may be complete or incomplete, as shown. Then there


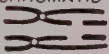

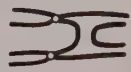




BREAKS (INITIAL BREAKS)	ISOCHROMATID BREAK	TWO CHROMATID BREAKS
		
COMPLETE FUSION (FUSED PROXIMALLY AND DISTALLY)	SU 	COMPLETE 
INCOMPLETE FUSION INCOMPLETE PROXIMALLY	NUp 	INCOMPLETE PROX 
INCOMPLETE FUSION INCOMPLETE DISTALLY	NUd 	INCOMPLETE DISTAL 
RATIO OF $\frac{\text{INCOMPLETE FUSION}}{\text{COMP} + \text{INC FUSION}}$	$\frac{\text{NUp} + \text{NUd}}{\text{SU} + \text{NUp} + \text{NUd}}$	$\frac{\text{INCP} + \text{INCD}}{\text{COMP} + \text{INCP} + \text{INCD}}$

Fig. 2 Equivalence of isochromatid and chromatid-chromatid interchange fusion classes.

is a complete fusion where again both ends are fused as for the isochromatid SU class, or it may be incomplete in one of two ways — proximally or distally.

In these exchange aberrations, both broken ends may fuse; or only the left-hand or the right-hand end. I think you can see that these classes are in fact identical with the similar isochromatid classes. The last isochromatid class, unfused both proximally and distally, is not detectable in the exchange aberrations, of course, for such an event would not result

in an exchange aberration at all, but only in two independent chromatid aberrations.

Miss Johnston and I did examine cells which had been irradiated in oxygen or in nitrogen for these exchange fusion classes. A test can be made of the method of analysis in this way. If there is a relation between the way the fusibility of isochromatid and chromatid breaks are affected, the fusion frequency should be the same for isochromatid aberrations as for the exchanges. As a matter of fact, the frequencies of these two are the same. From cells which were irradiated in oxygen, the incompleteness of the isochromatid aberrations was about 18% ; and it was 18% for exchanges. For nitrogen, the isochromatid incompleteness was also 18%. Therefore, the fusibility of the broken ends that produce isochromatid aberrations must be the same in oxygen and in nitrogen. The differential yield of isochromatid aberrations, which is the experimentally observed fact, cannot be explained on the basis of an effect on fusibility.

However, the exchange aberrations produced by irradiation in nitrogen were about twice as incomplete (i.e., the frequency of incompleteness was about twice as great) as those produced in oxygen. So, apparently, there is a difference in the fusibility of chromatid breaks produced in nitrogen and in oxygen. But observe that the incompleteness is in the wrong direction to fit the hypothesis that the twofold increase in aberration yield in going from nitrogen to oxygen is due to an effect on fusion ; the hypothesis requires that fusion be *less*, not more, in oxygen than in nitrogen. So for these aberrations, the hypothesis would be wrong by a factor of four.

GILES: I should like to recall, in connection with what Dr. Conger has said, that I mentioned very briefly in the paper that we have made preliminary observations of this same sort which agree very well with those of Dr. Conger. Our data are certainly not as extensive as his, but they agree in showing no difference in oxygen and nitrogen in failure of reunion.

I would recall also that the same type of observation was made in connection with the early fast neutron-X-ray com-

parisons, where it was at one time thought possible that differences in various chromatid aberration ratios might be caused, in neutrons, by an effect on rejoining rather than on breakage.

GLASS: Somebody said that I read Dr. Swanson's paper with a good deal of conviction. I just want it to be clear that I do not agree completely with his conclusions. I have some data that I think rather better support the point of view that Dr. Baker has just presented. My data, like his, were obtained on *Drosophila*. The dominant mutations that produce *Minute* bristles in *Drosophila* are very common and are well known to be associated with small deficiencies in the great majority of cases. The dosage curves for *Minutes* are practically identical for treated males and females over a range of 500–4000 r units. This relation shows that chromosomes are broken by X radiation with the same frequency in oocytes as in spermatozoa. The *Minutes* result from the same type of intrachromosomal breakage and fusion that Dr. Baker was talking about, and for which he predicted that there would be no oxygen effect.

The particular studies that we have carried out with oxygen on *Minutes* did not represent a comparison of the respective frequencies in air and in nitrogen, but instead in air and in pure oxygen. Females given a dose of 2000 r showed a very slight increase in frequency of *Minutes* in the shift from 21 to 100% oxygen. There was a practically identical curve for the males given 2000 r of X rays. The curve is very flat.

On the other hand, translocations, which of course involve breakage in different chromosomes, are very rare in the females, whereas you get them in relatively high proportions in the males. Hence it is interesting to see what effect oxygen has on the frequency of translocations in *Drosophila melanogaster*.

The results we have obtained — based on about 900 gametes tested in each category — are rather different from those which Dr. Baker has published for *Drosophila virilis*. In the females, the translocation frequency in pure oxygen shows

practically no increase over that in air. There is very little frequency in either case. But for the males, the frequency (in two successive experiments that agreed very consistently) changed from about 8% in air to 14.7% in pure oxygen for a dose of 2000 r. This shows a very decided oxygen effect in interchromosomal changes; and, so far as the *Minutes* show, very little oxygen effect on the intrachromosomal type of breakage and restitution phenomenon.

KIMBALL: The main theme of this session has been the biological nature of the processes which are sensitive to oxygen present during irradiation. The chemical events in which molecular oxygen is involved quite properly have been subordinate. However, attention should be called to work with bacteriophage, DNA, and enzymes reported in a symposium on aftereffects, in the *British Journal of Radiology* (1954, vol. 27). For all three entities, the immediately detectable effects of X irradiation are oxygen insensitive or are even increased by the absence of oxygen while the aftereffects, which sometimes require a number of hours for full expression, are decreased by the absence of oxygen. I feel that this is of considerable potential interest since a number of the results presented in this session suggest to me that the timing of chromosomal events may not be the same in material irradiated in oxygen and in nitrogen.

Two mechanisms have been found for the oxygen sensitivity of the aftereffect. In bacteriophage, and perhaps to some extent in DNA, the material is sensitized to  $H_2O_2$  and other agents by the action of reducing radicals. Oxygen acts by influencing the amount of  $H_2O_2$  formed, and this substance reacts somewhat slowly with the sensitized material to bring about an aftereffect. The other mechanism, found for both DNA and pepsin, is the formation of unstable states in the macromolecule by the action of oxidizing radicals. Oxygen acts in the formation of the oxidizing radicals, and the aftereffect is not caused by long-lived, slowly reacting, small-molecular products of irradiation. In *Paramecium aurelia* I have been able to demonstrate that  $H_2O_2$  is not responsible



in any way for the increased mutation found when X irradiation is carried out in air rather than nitrogen. It seems quite probable that this conclusion can be extended to the production of chromosome aberrations and mutations in other organisms, especially when the small quantities of peroxide which can be formed within the cell and the slowness of its reactions with substances such as DNA are taken into account. This makes it tempting to consider the second explanation for the after-effect as applying to chromosomes also but this would be a large extrapolation beyond our present fragmentary information and does not, any more than the other hypotheses so far suggested, provide a single, unified explanation of the results.

In fact, it seems rather unlikely to me that such unified explanation will be forthcoming. Present work suggests that oxygen affects in some manner the number of detectable breaks, the amount of recognizable reunion, the duration of the time over which breaks remain available for recombination, and the rate of progress of cells through mitosis. This suggests that no hypothesis which proposes a single biological effect as the target for the oxygen-radiation interaction will be successful in encompassing all the data.

BEATTY: Some work which I am finishing on the speed of the first microspore division in *Tradescantia* might have an important role in the interpretation of Dr. Swanson's work. At 30°C. very early prophase requires 24 hours for completion; early prophase, 4 hours; mid-prophase, 3 hours; late prophase, 3 hours; and the remaining stages a half-hour for each. If buds having microspores in division are given 200 r at 50 r per minute, a critical point in mid-prophase will be found in which cells having passed this point will proceed through division, forming clumped metaphases and anaphases. Cells not having reached this critical point, both mid-prophase and early prophase stages, will revert to very early prophase. In addition to this reversion, there is a delay in the very early prophase stage. As the cells recover from the effects of radiation, it is believed that there is a reshuffling of the mid-, early,



and very early prophase stages. The reversion and delay in division is not nearly so great in helium as it is in oxygen.

WOLFF: I noticed that Dr. Giles, and also Dr. Glass in reading Dr. Swanson's paper, referred to some of my earlier experiments in which I interpreted the results as indicating that oxygen or anoxia has affected chromosome restitution and not breakage. However, since then Atwood and I have run a considerable number of experiments on dosage fractionation effects in *Vicia*, which have led us to change our stand.

It seems that the effect of anoxia, during irradiation, on restitution is independent of the effects on breakage; and no matter how long the breaks stay open, the same numbers of aberrations are obtained. So we are led to believe that the effect of oxygen or anoxia on *Vicia* is an effect on the primary breakage and not on restitution. There is indeed an effect on restitution, but this does not influence aberration yield.

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